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# TOTAL BLOOD CELL VOLUME DETERMINATION IN NORMAL AND ANAEMIC DOGS USING METHAEMOGLOBIN AS TRACER (*)

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ACCORDING to some authors, the colorimetric determination of plasma volume as a preliminary step to obtain the total blood volume calculated through the jugular haematocrit gives higher values than the total blood volume obtained with combined methods, which detect the plasma and red cell volumes separately. Consequently, for studies on the distribution of plasma and red cells, a total blood volume estimation, obtained by adding the results of red cell and plasma volume determinations, is essential.

Of the different techniques for determining the total red blood cell volume using carbon monoxide, red cells tagged with Heinz corpuscles (7), or radioactive substances, the most simple and reliable are the latter. Not being able as yet to use radioactive procedures in our laboratory, we developed the present method with the object of using it on dogs during experimental shock. It is a method for experimental work with no claims for its application in practice nor for other types of determinations. It could be considered as an improvement of the method described by Moore *et al.* (6).

## METHODS

The method used in this work is based on the well known fact that the red cell does not exchange its haemoglobin content during its life cycle. The median corpuscle haemoglobin concentration is a haematological constant in normal animals and varies slowly even in pathological conditions. A method based on such a constant should be less subject to error than others, taking into consideration the ratio plasma/red cells in the calculations of the total blood volume, on account of the variation of this ratio throughout the circulatory system. There is proportionally more plasma than cells in the capillaries than in the great vessels, but the ratio red cell volume/haemoglobin

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content is constant, the constituents of the blood cells remaining the same during the circuit of the blood from the capillaries to the great vessels and vice-versa.

The general idea of the method consists in injecting a known amount of red cells in which the haemoglobin has been chemically transformed into methaemoglobin and, after a period of time —to allow complete mixing of the injected erythrocytes with the red cells of the animal—, to determine the methaemoglobin content of the blood and thus by calculation obtain the total red cell volume, which is inversely proportional to the amount of dilution of the injected mathaemoglobin.

Two subsidiary considerations should be taken into account: the determination of the physiological methaemoglobinaemia and the rate of conversion of the injected methaemoglobin into haemoglobin in the animal during the period of the determination.

To determine the plasma volume, Evans blue dye (T-1824) was used, both determinations —plasma and red cell volumes— being done at the same time.

For clarity the method will be divided into five different procedures: 1) Physiological reduction of methaemoglobin. 2) Preparation of the red cells with methaemoglobin. 3) Determination of methaemoglobin in the circulating blood. 4) Injection and sample collection of the red cells using methaemoglobin and Evans blue dye. 5) Calculations and an example of the determination.

TABLE I

| Dog number | Body weight (kg) | Circulating haemoglobin (g) | Injected methaemoglobin (g) | Methaemoglobin (%) Minutes 5 | 10 | 20 | 30 | 60 |
|---|---|---|---|---|---|---|---|---|
| 658 | 7.8 | — | 5.5 | 8.2 | 7.3 | 5.5 | 5.4 | 5.2 |
| 658A | — | 10.4 | — | 11.2 | 11.0 | 10.6 | 10.0 | 9.2 |
| 656 | 8.2 | 11.8 | 5.3 | 8.1* | 8.9 | 7.6 | 7.1 | 7.0 |
| 662 | 9.0 | 11.0 | 6.2 | 7.8 | 7.3 | 6.9 | 6.7 | 6.2 |

* Five minute determinations were not reliable, probably on account of insufficient mixing of the injected cells with the cells of the recipient.

1) *Physiological reduction of methaemoglobin.*

It is known that methaemoglobin is slowly reduced within the body to haemoglobin, which recovers its capacity to transport oxygen as oxyhaemoglobin. The rate of this reduction had to be known before any attempt to determine blood volumes could be made. Four dogs were submitted to this test, samples of blood being taken for the methaemoglobin determination with the same interval of time (table I).

The analysis of the methaemoglobin reduction in the interval between five and sixty minutes shows an exponential shaped curve, but for the red cell volume determination, however, the curve limits between ten and twenty minutes could be considered as linear for the correction effect due to the progressive reduction of methaemoglobin during this period of time.

2) *Preparation of red cells with methaemoglobin.*

A normal dog is bled from the jugular vein using sodium citrate as anti-coagulant. The amount taken is proportional to the body weight of the dog in which the red cell volume is to be determined (5ml of blood per kg of body weight). An excess of sodium nitrite is now added to the blood as a spur of solid salt. The blood is shaken

to dissolve the nitrite and allowed to stand three to four hours till practically all the haemoglobin has been converted into methaemoglobin. The blood is centrifuged, the supernatant discarded and the packed cells washed several times with 200 ml of saline, till the supernatant shows no traces of nitrite, i.e. when no blue color appears on a filter paper soaked with a mixture of starch and potassium iodide. The supernatant is discarded and the packed cells resuspended to half volume with saline. They are now ready to be injected. To determine the amount of MHb. take 0.1 ml and complete to 6 ml with distilled water. Find the optical extinction at 618 $m\mu$. The result (g/ml) is obtained by multiplying its extinction by 100 and dividing by the specific extinction coefficient of MHb. at 618 $m\mu$ (200) and by the dilution (6/0.1).

3) *Determination of methaemoglobin in the circulating blood.*

The method for determining blood methaemoglobin colorimetrically has been described elsewhere (2). We give here a mere schematic procedure. All the colorimetric work has been done with a model DU Beckmann spectrophotometer*.

1) 3 ml of blood is collected from the jugular vein into a tube containing a mixture of oxalate salts (0.12 ml of a solution of potassium oxalate 2 % and ammonium oxalate 3 %).

2) To find the total haemoglobin of the sample (oxyhaemoglobin plus methaemoglobin) take 0.02 ml of blood in 3 ml distilled water. Add a spur of potassium ferricyanide (for conversion of oxyhaemoglobin into methaemoglobin) and afterwards a spur of potassium cyanide (for conversion of methaemoglobin into cyanmethaemoglobin). Find the light extinction (1 cm light path cube) at 540 $m\mu$. Total haemoglobin (g per 100 ml blood) is found by multiplying the extinction by 21.7 (Constant derived from the specific extinction of cyanmethaemoglobin at 540 $m\mu$ — 0.00145 — multiplied by 100 and the blood dilution correction — 3/0.02).

3) To find the methaemoglobin content, take 2 ml of blood, centrifugate at 3000 revolutions per minute, for ten minutes. Discard the supernatant and add 3 ml of distilled water. Shake violently and wait five minutes for complete haemolysis. Add 40 mg of sodium chloride. Shake. Centrifuge at 3000 revolutions per minute, for 15 minutes. Carefully remove most of the supernatant which will be the sample to be determined.

4) Before proceeding, the total haemoglobin of this sample should be determined: 0.1 ml of the haemolysed supernatant is put in 2.9 ml of distilled water. Haemoglobin of the sample (g per 100 ml) is obtained by multiplying the light extinction obtained at 540 $m\mu$ by 4.35 (constant derived from 0.00145 × 100 × 3/0.1).

5) Find the extinction of the sample to be determined at 618 $m\mu$ (this result we called $E_o$). Add to the sample still in the spectrophotometer cube a spur of potassium cyanide and stir with a thin rod. Find the new extinction at 618 $m\mu$ (result called E).

6) Calculation:

$$\text{Methaemoglobin in relation to total haemoglobin (\%)} = \frac{(E_o - E)\ 10000}{\text{Sample Hb.} \times 140^{**}}$$

$$\text{Methaemoglobin (g per 100 ml of blood)} \ldots\ldots = \frac{\text{MHb. (\%)} \times \text{Total Bb}}{100}$$

4) *Injection and sample collection of the red cells using methaemoglobin and Evans blue dye.*

1) The jugular vein is punctured and 10 ml of blood taken and put in a flask with an oxalate mixture (0.4 ml of the mixture) in dry state. 5 ml is to be used for plasma volume, 3 ml for physiological methaemoglobin and 2 ml for current haematological data.

2) The red cells with methaemoglobin are injected through the jugular vein, the syringe washed with saline, and this saline injected also. A stop watch is started at

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** Constant corresponding to unities of light extinction (MHb specific extinction coefficient minus CyanMHb. extinction coefficient at 618 $m\mu$).

the end of the injection. When total blood determinations were desired as soon as the injection of cells was finished, a solution of Evans Blue was injected (5 ml of a 0.1 g % of Evans Blue for dogs between 6 and 10 kg).

3) Ten minutes after the injection of cells, 10 ml of blood were removed through the needle, *which should have remained in the jugular*, and put in a flask containing the appropriate amount of oxalate mixture (0.4 ml). 5 ml of blood is centrifuged, the supernatant taken and the light extinction determined at 618 $m\mu$ against the plasma taken before the injections, to obtain the total plasma volume. The other 5 ml will be used for the first MHb determination ($MHb_{10}$).

4) Twenty minutes after the injection another 5 ml of blood is taken and the second MHb determination made ($MHb_{20}$).

5) *Calculation and an example of the determination. Calculation of total plasma volume.*

The specific extinction coefficient of Evans blue at 618 $m\mu$ is 50 000, a solution of 0.00002 g/ml giving an extinction of 1.0. Thus knowing the amount of dye injected (I) and the plasma extinction (PE) after the mixing of the dye in the circulation, the amount of total plasma is given by the formula:

$$\text{Plasma volume (ml)} = \frac{50000 \times \text{I (g)}}{\text{PE}}$$

*Calculation of total red cell volume.*

The MHb. value to calculate the total red cell volume should be corrected for the reduction within the body and also substracted by the physiological MHb. of the animal. Thus the MHb. value for the ten minutes sample is corrected according to the formula:

$$\text{MHb.}_1 = \frac{\text{MHb.}_{10}}{\text{MHb.}_{20}} \times \text{MHb.}_{10}$$

and the final result found after:

$$\text{MHb.} = \text{MHb.}_1 - \text{MHb.}_0$$

where $MHb._0$ stands for the physiological methaemoglobinaemia before the test.

Knowing now the amount of MHb. injected ($MHb._I$), the corrected MHb. concentration in the blood after the mixing of the injected red cells with the cells of the recipient (MHb.) and the mean corpuscular concentration (MCC) the total red cell volume si calculated by the formula:

$$\text{Total red cell volume (ml)} = \frac{\text{MHb.}_\text{I}\ \text{(g)} \times 10000}{\text{MCC}}$$

To illustrate this rather involved procedure a protocol of a typical determination is given:

*Dog 706.* — Weight: 8.7 Kg. Haemoglobin: 8.3 g/100 ml. Jugular haematocrit: 26 %. Mean corpuscular concentration: 32 %.

| | *Optical extinction at 618* | *Calculation* |
|---|---|---|
| *Plasma volume* | | |
| Injection of 5 ml of a 0.1 % solution of Evans blue (5 mg) | 0.520 | $PV = \frac{50000 \times 0.005}{0.520} = 480$ ml |
| *Red cell volume* | | |
| Physiological methaemoglobinaemia | | |
| Sample haemoglobin | 1.55 | $\text{MHb.} = \frac{(0.84 - 0.80) \times 10000}{(1.55 \times 4.35) \times 140} = 0.4$ % |
| $E_o$ | 0.84 | |
| E | 0.80 | |

| | | |
|---|---|---|
| Methaemoglobin injected (20 ml) | 0.500 | $MHb. = \frac{0.500 \times 0.6 \times 100}{200} = 0.15$ (g/ml) |
| MHb. (10 minutes sample) | | |
| Sample haemoglobin | 0.500 | $MHb. = \frac{(0.46 - 0.34)\ 10000}{(0.5 \times 4.35)\ 140} = 3.9\ \%$ |
| $E_o$ | 0.460 | |
| E | 0.340 | |
| MHb. (20 minutes sample) | | |
| Sample haemoglobin | 0.650 | $MHb. = \frac{(0.68 - 0.54)\ 10000}{(0.65 \times 4.35)\ 140} = 3.5\ \%$ |
| $E_o$ | 0.680 | |
| E | 0.540 | |
| Correction for MHb. reduction | | $MHb. = \frac{3.9 \times 3.9}{3.5} = 4.3\ \%$ |
| Correction for physiological methaemoglobinaemia | | $MHb. = 4.3 - 0.4 = 3.9\ \%$ |
| Red cell volume | | Red cell vol. $= \frac{3.0 \times 10000}{3.9 \times 32} = 240$ ml |

*Reliability of the method*

To ascertain the consistency of the method five determinations were performed on the same dog within a period of a week. These results are presented in Table II.

TABLE II

*Five determinations of blood volume in the same animal. Dog 706 (8.7 kg)*

| Day | Hemoglobin g/100 ml | Jugular haematocrit % | Plasma volume ml | MHb. injected g | MHb. (%) Minutes 10 | MHb. (%) Minutes 20 | Red cell volume ml | Total blood volume ml | Body haematocrit % | Percentual difference between body and jugular haematocrits % |
|---|---|---|---|---|---|---|---|---|---|---|
| V-8 | 8.3 | 26 | 480 | 3.0 | 3.9 | 3.6 | 247 | 727 | 34 | + 31 |
| V-9 | 9.2 | 28 | 510 | 2.8 | 3.3 | 3.1 | 253 | 760 | 33 | + 18 |
| V-14 | 9.0 | 28 | 531 | 2.4 | 3.2 | 2.8 | 234 | 765 | 31 | + 10 |
| V-15 | 9.2 | 31 | 530 | 3.0 | 3.6 | 3.5 | 265 | 795 | 33 | + 6 |
| V-17 | 9.8 | 30 | 520 | 1.5 | 2.7 | 2.3 | 279 | 799 | 35 | + 17 |

## RESULTS AND DISCUSSION

Table II presents results of red cell and plasma volumes done on the same animal within five days to ascertain the consistency of the results. The plasma volume dye method has an accuracy of ± 5 % according to data found in the literature. The results in Table II show a discrepancy of ± 7 %, practically the same as found by other authors with this method. The red cell volume showed a variation of ± 9 %, a result that is similar to determinations by other methods. Considering that the determinations were made on different days in which the plasma and red

TABLE III

| Number | Body weight kg | Haemoglobin g | Jugular haematocrit % | Plasma volume ml | Red cell volume ml | Total blood volume ml | Relation Blood volume/Body weight % |
|---|---|---|---|---|---|---|---|
| | | | NORMAL DOGS | | | | |
| 693 | 6.3 | 12.4 | 44 | 292 | 191 | 483 | 7.7 |
| 678 | 8.2 | 13.4 | 42 | 301 | 321 | 622 | 7.6 |
| 679 | 6.8 | 13.8 | 45 | 328 | 451 | 779 | 11.4 |
| 689 | 7.0 | 11.0 | 38 | 471 | 338 | 809 | 11.6 |
| 698 | 8.3 | 11.2 | 37 | 333 | 156 | 489 | 5.9 |
| 699 | 5.5 | 11.4 | 42 | 250 | 259 | 509 | 9.2 |
| 695 | 9.1 | 11.2 | 41 | 471 | 330 | 801 | 8.8 |
| 670 | 12.2 | 12.2 | 43 | 500 | 235 | 735 | 6.1 |
| 671 | 6.7 | 11.0 | 41 | 330 | 187 | 517 | 7.7 |
| 672 | 12.8 | 12.8 | 40 | 531 | 416 | 947 | 7.4 |
| 694 | 8.1 | 13.2 | 45 | 357 | 153 | 510 | 8.3 |
| 696 | 6.6 | 13.6 | 42 | 274 | 318 | 592 | 9.0 |
| 699 | 7.3 | 11.4 | 45 | 387 | 309 | 696 | 9.5 |
| 703 | 7.9 | 12.2 | 41 | 337 | 375 | 712 | 9.0 |
| | | | ANEMIC DOGS | | | | |
| 675 | 8.3 | 10.8 | 35 | 416 | 285 | 701 | 8.4 |
| 677 | 7.7 | 6.8 | 24 | 396 | 235 | 631 | 8.2 |
| 692 | 9.0 | 8.4 | 33 | 563 | 253 | 816 | 9.1 |
| 683 | 9.3 | 9.8 | 34 | 434 | 172 | 606 | 6.5 |
| 704 | 10.1 | 7.2 | 21 | 806 | 133 | 939 | 9.3 |
| 674 | 9.5 | 6.8 | 27 | 581 | 165 | 746 | 7.9 |
| 676 | 7.7 | 6.2 | 26 | 409 | 151 | 560 | 7.3 |
| 695 | 8.2 | 9.4 | 33 | 426 | 181 | 607 | 7.4 |
| 697 | 8.3 | 10.6 | 36 | 446 | 145 | 591 | 7.1 |
| 698 | 8.7 | 10.8 | 35 | 416 | 285 | 701 | 8.1 |
| 700 | 8.9 | 7.0 | 23 | 487 | 181 | 668 | 7.5 |
| 701 | 11.0 | 10.8 | 36 | 617 | 280 | 897 | 8.2 |
| 702 | 5.9 | 6.0 | 20 | 454 | 92 | 546 | 9.2 |

of the method, the values found for the variations of the techniques should be regarded as satisfactory. The relationship between jugular cell volume of the dog could have varied, as well as the known inaccuracy and body haematocrits in the calculation of which the errors of both methods are added showed a percentage difference error of ± 15 %

Table III shows the results in 14 normal dogs (Haemoblobin 11.0 g/100 ml or over and haematocrit 37 % or over) and 13 anaemic dogs (Haemoglobin below 11 g/100 ml and haematocrit below 37 %). The combined methods worked in a comparable way independent of the size of the dog: normal dog 693, of 6.3 kg, gave a total blood volume of 483 ml (radio blood volume/body weight = 7.4) ; dog 672, of 12.8 kg, gave 947 ml (ratio = 7.4) ; anaemic dog 701, 11.0 kg, gave 897 ml (ratio = 9.2) ; and anaemic dog 702, of 5.9 kg, gave 546 ml (ratio = 9.2).

The usual test of the accuracy of a blood volume determination method is to compare its results with those found by other methods. For

TABLE IV

| Author | Methods and averages of ratio BV/BW | Jugular haematocrit % | Body haematocrit % | Percentual difference | Ratio total blood volume/body weight |
|---|---|---|---|---|---|
| Hopper, J., Tabor, H. and Winkler, H. | Carbon monoxide and Evans blue (T-1824) Normal dogs: 10.5 ± 3.1 Anemic dogs 10.5 ± 1.0 | 57 | 61 | — 7 | 10.0 |
| | | 45 | 53 | + 16 | 13.6 |
| | | 44 | 43 | — 3 | 8.7 |
| | | 41 | 34 | — 4 | 9.2 |
| | | 32 | 35 | — 8 | 10.0 |
| | | 46 | 43 | — 7 | 9.5 |
| | | 47 | 47 | 0 | 10.0 |
| | | 50 | 47 | — 6 | 11.2 |
| | | 41 | 39 | — 5 | — |
| | | 59 | 60 | + 2 | 9.5 |
| | | 31 | 31 | 0 | 11.0 |
| | | 30 | 27 | — 10 | 10.8 |
| | | 38 | 37 | — 3 | 11.0 |
| | | 38 | 38 | 0 | 9.5 |
| | | 28 | 25 | — 10 | 10.4 |
| | | 41 | 39 | — 5 | 12.6 |
| | | 41 | 39 | — 5 | 11.4 |
| Hahn, P., Balfour, W. et. al. | Radiactive iron and brillant vital red Normal dogs: 7.7 ± 1.3 Anemic dogs: 9.1 | 39 | 34 | — 13 | 7.9 |
| | | 51 | 48 | — 6 | 8.9 |
| | | 45 | 34 | — 25 | 6.4 |
| | | 50 | 40 | — 20 | 6.5 |
| | | 54 | 47 | — 13 | 7.6 |
| | | 40 | 34 | — 15 | 7.7 |
| | | 45 | 42 | — 7 | 8.9 |
| | | 21 | 19 | — 9 | 9.1 |
| Root, W., Roughton, F. and Gregersen, M. | Carbon monoxide and Evans blue Normal dogs: 10.2 ± 2.9 Anemic dogs: 9.9 ± 0.1 | 33 | 36 | + 9 | 10.0 |
| | | 35 | 35 | 0 | 9.8 |
| | | 53 | 51 | — 4 | 9.9 |
| | | 45 | 46 | + 2 | 11.4 |
| | | 44 | 45 | + 2 | 11.1 |
| | | 50 | 48 | — 4 | 7.3 |
| | | 50 | 50 | 0 | 11.5 |
| Krieger, H. Storaasli, J. Friedell, H. and Haldon, W. | Radiactive phosphorus and Evans blue Normal dogs: 9.6 ± 1.4 Anemic dogs: 11.0 ± 1.4 | 48 | 43 | — 11 | 10.0 |
| | | 39 | 39 | 0 | 9.2 |
| | | 38 | 47 | + 23 | 9.7 |
| | | 32 | 53 | + 65 | 12.4 |
| | | 39 | 42 | + 8 | 9.9 |
| | | 33 | 38 | + 14 | 10.3 |
| | | 44 | 45 | + 2 | 10.0 |
| | | 32 | 36 | + 12 | 11.0 |
| | | 49 | 42 | — 14 | 9.1 |
| | | 31 | 36 | + 15 | 10.3 |

| | | | | | |
|---|---|---|---|---|---|
| Krieger, H., Storaasli, J. Friedell, H. and Halden, W. | Radiactive phosphorus and radiactive iodine. Normal dogs: 9.3 ± 0.7. Anemic dogs: 9.7 ± 1.7 | 48 | 45 | — 6 | 9.6 |
| | | 39 | 39 | 0 | 9.2 |
| | | 38 | 48 | + 26 | 9.5 |
| | | 32 | 57 | + 78 | 11.4 |
| | | 39 | 43 | + 10 | 9.5 |
| | | 33 | 41 | + 24 | 9.5 |
| | | 44 | 48 | + 9 | 9.5 |
| | | 32 | 43 | + 34 | 9.1 |
| | | 49 | 45 | — 8 | 8.6 |
| | | 31 | 41 | + 32 | 9.1 |
| Smith, H., Arnold, H. and Whipple, G. | Carbon monoxide and Haemoglobin. Normal dogs: 9.4 ± 2.6 | 45 | 44 | — 2 | 12.0 |
| | | 40 | 38 | — 5 | 8.6 |
| | | 46 | 35 | — 24 | 8.1 |
| | | 41 | 40 | — 2 | 10.0 |
| | | 53 | 58 | + 9 | 9.7 |
| | | 52 | 45 | — 13 | 8.4 |
| | | 49 | 44 | — 10 | 9.4 |
| | | 62 | 60 | — 3 | 9.2 |
| | | 48 | 42 | — 12 | 10.0 |
| | | 59 | 52 | — 12 | 9.3 |
| | | 59 | 54 | — 9 | 8.5 |
| | | 50 | 45 | — 10 | 9.9 |
| | | 46 | 45 | — 2 | 10.1 |
| | | 58 | 50 | — 14 | 8.7 |
| Cruz, W. O. and Oliveira, H. P. | Methaemoglobin and Evans blue. Normal dogs: 8.5 ± 3.1 | 44 | 40 | — 9 | 7.7 |
| | | 42 | 52 | + 24 | 7.6 |
| | | 45 | 58 | + 42 | 11.4 |
| | | 38 | 42 | + 11 | 11.6 |
| | | 37 | 32 | — 13 | 5.9 |
| | | 42 | 51 | + 21 | 9.2 |
| | | 41 | 41 | 0 | 8.8 |
| | | 43 | 32 | — 25 | 6.1 |
| | | 41 | 36 | — 12 | 7.7 |
| | | 40 | 44 | + 10 | 7.4 |
| | | 45 | 30 | — 33 | 8.3 |
| | | 42 | 54 | + 28 | 9.0 |
| | | 45 | 44 | — 2 | 9.5 |
| | | 41 | 52 | + 27 | 9.0 |
| Cruz, W. O. and Oliveira, H. P. | Methaemoglobin and Evans blue. Anemic dogs 8.0 ± 1.5 | 35 | 41 | + 17 | 8.4 |
| | | 33 | 31 | — 6 | 9.1 |
| | | 34 | 28 | — 18 | 6.5 |
| | | 21 | 14 | — 33 | 9.3 |
| | | 27 | 22 | — 19 | 7.9 |
| | | 26 | 27 | + 4 | 7.3 |
| | | 24 | 37 | + 54 | 8.2 |
| | | 33 | 30 | — 9 | 7.4 |
| | | 36 | 25 | — 30 | 7.1 |
| | | 35 | 41 | + 17 | 8.1 |
| | | 23 | 27 | + 17 | 7.5 |
| | | 36 | 31 | — 14 | 8.2 |
| | | 20 | 17 | — 15 | 9.2 |

that purpose the ratio total blood volume/body weight constitutes a good basis for a comparative analysis of the results. Table IV presents this ratio in normal and anaemic dogs according to several authors. Normal dogs varied between 7.9 and 10.5 as averages in seven papers quoted. From these seven groups of dogs the maximum variation found between each group was ± 3.1 and the minimum variation ± 1.3. Considering all groups together the total variation of the relationship blood volume/body weight was between 6.4 and 13.6. For anaemic dogs the higher was 11.0 ± 1.4 and the lower 9.3 ± 0.7, the total variation being in all dogs together between 8.6 and 12.4. Our results for the normal dog (8.5 ± 3.1) are a little lower than the average of other authors, and in anaemic dogs (8.0 ± 1.5) are definitely lower than those found in the literature.

A problem of much concern to many workers in blood volume studies is the relative concentration of plasma and red blood cells throughout the circulatory system. Many authors have pointed out that the concentration of red cells should be less in the capillary bed than in the great vessels, to explain satisfactorily the results of their work ([3, 9]). This "haemodilution" in the peripheral bed has been definitely shown by direct microscopic observation of the capillaries *in vivo* ([1]). On the other hand, nowadays some authors defend the opposite thesis, arguing that this haemodilution occurs in a minimal fraction of the blood, and consequently is not important enough to cause appreciable difference in the haematocrit of a great vessel as compared with the haematocrit of the whole blood ([8]).

It is odd that these authors do not compare their own data related to this problem. We have collected the results taken from the papers of several authors in table IV, in which the jugular haematocrit, the haematocrit of the whole body (body haematocrit) and the percentual differences between them are found. The differences found in these papers are striking. Hopper *et al.* ([4]), and Root *et al* ([8]), found very little difference in blood concentration: in 17 dogs of the first paper a variation of + 16 % and — 10 % is quoted and a discrepancy of 9 % in only one case out of seven in the second paper. This similarity of the jugular and body haematocrits is firmly denied by the others authors. Hahn *et al.* ([3]) and Smith *et al.* ([9]), with a single exception out of 22 dogs found haematocrit to be consistently lower than the jugular haematocrit (a maximum difference of — 25 % was observed). Using radioactive substances Krieger and collaborators ([5]) found, on the contrary, as a general rule, a higher body haematocrit than the jugular haematocrit (even as high as + 78 %) in ten dogs examined. The present paper shows differences in both directions: variations from —33 % to + 42 % have been observed in normal dogs, while in anaemic dogs figures between — 33 % to + 54 % have been found.

It is not easy to explain satisfactorily the discrepancy of the results of blood volume determinations found in the literature. Great variation with the same method and the same authors seems nevertheless to indicate that the concentration of blood could vary in the same animal under different physiological conditions. Possibly the amount of exercise previous to the blood volume determination, or the length of time the animal is

kept in an artificial position, or some other cause not yet considered, could interfere with the distribution of the red cells and plasma in the circulatory system. The question of whether these conditions have or have not a relationship with the distribution of red cells in the large and small vessels, seems to us worthy of study.

## SUMMARY

A method is described for the determination of the total red cell volume in dogs, by injection of red cells in which a previous transformation of its haemoglobin into methaemoglobin has been chemically performed. Taking into consideration the physiological methaemoglobinaemia and the reduction of the injected methaemoglobin within the body, the degree of dilution of the injected red cells in the blood stream is proportional to the total red cell volume.

The error of the method is around $\pm$ 9 %. Combined with the dye method (Evans blue, T - 1824) it gives for the ratio total blood volume/body weight, $8.5 \pm 3.1$ in fourteen normal dogs, while thirteen anaemic dogs give $8.0 \pm 1.5$ for this same ratio. These results agree with the variation found in the literature.

The problem of the relationship between the jugular haematocrit and the haematocrit of the whole body has been taken into consideration, values have been calculated from several papers and the results compared and analysed.

## BIBLIOGRAPHY

(1) CHAMBERS, R., ZWEIFACH, B. W., LOWENSTEIN, B. E.: *Ann. Surg.*, 1944, *120*, 791.
(2) CRUZ, W. O.: *Acta Haemat.*, 1951, *6*, 367.
(3) HAHN, P. F., BALFOUR, W. M., ROSS, J. F., BALE, W. F., WHIPPLE, G. H.: *Science*, 1941, *93*, 87.
(4) HOPPER, J., TABOR, H., WINKLER, A. W.: *J. clin. Invest.*, 1944, *23*, 628.
(5) KRIEGER, H., STTORAASLI, J. P., FREDELL, H. L., HOLDEN, W. D.: *Proc. Soc. exper. Biol.*, N. Y., 1948, *68*, 511.
(6) MOORE, J. C., SHADLE, O. W., LAWSON, H. C.: *Amer. J. Physiol.*, 1948, *153*, 322.
(7) NIZET, A.: *Quart. J. exp. Physiol.*, 1948, *34*, 123.
(8) ROOT, W. S., ROUGHTON, F. J. W., GREGERSEN, M. I.: *Amer. J. Physiol.*, 1946, *146*, 739.
(9) SMITH, H. P., ARNOLD, H. R., WHIPPLE, G. H.: *Amer. J. Physiol.*, 1921, *56*, 336.

# QUANTITATIVE VARIATIONS OF THE NEUTRAL 17-KETOSTEROIDS IN ALLOXAN-DIABETIC RABBITS

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CARBOHYDRATE metabolism is controlled in the normal body to a considerable degree by the adrenal glands, both adrenaline and glucocorticoids being active in this control. The rôle played by the adrenal glands both in experimental and clinical diabetes also has been studied. It has been shown that diabetes diminishes after adrenalectomy (Long, 1936) and that a diabetic patient with Addison's disease requires much less insulin than usually (Bloomfield, 1939). Tumors or hyperplasia of the adrenals may cause hyperglycemia and diabetes resistant to insulin.

On the other hand it is known that the neutral 17-ketosteroids (17-KS) are the metabolic end products of the adrenal steroid hormones (corticoids), of those of the testis and probably also of the *corpus luteum*. The greater part of the corticoids are eliminated by the urine in form of inactive substances (Selye, 1949). Thus a sudden increase or an excessive decrease of corticoids in the body significantly modify elimination of 17-KS; their dosage in the urine procures a knowledge of the functional condition of the adrenals. The purpose of the present work was to study adrenal function in alloxan diabetes as evidenced by quantitative variations of urinary 17-KS in this experimental condition.

## METHODS

Salmon's technique for the determination of 17-KS was used (see details in Zañartu *et al.*, 1950). Titrations were performed in a Coleman spectrophotometer reading only at a wave length of 530 m$\mu$, without corrections. A correction formula has been given by various workers (Hamburger, 1948; Talbot *et al.*, 1942) but according to others (Egstrom and Mason, 1943) correction is not essential when purification with Girard's reagent is performed and most chromogens are thus eliminated. Under these conditions readings at both wave lenghts as 470 and 530 m$\mu$ are not essential either. As the urine of rabbits contains a large quantity of chromogens purification with Girard's reagent is fundamental.

Fourteen rabbits (6 males and 8 females, 1.7 to 3.0 kg) maintained on a mixed and complete diet were employed.

Normal values of urinary 17-KS were obtained by making repeated determinations in the same animal during more than one month. Diabetes was produced by

means of the usual technique of alloxan (Eastman-Kodak) administration; in one case alloxantine of our own preparation was used. Urine was collected in metabolic cages. After administration of alloxan, determinations were made in the urine collected during the first 24 hours. Afterwards determinations were performed every 48 hours; but all values are given in mg per 24 hours. Glycosuria was determined by the test of Fehling; blood sugar was determined by a modification of Shaffer-Hartmann's technique. Four animals were treated with protamine-zinc insulin (Squibb): one with 50 U and the remaining with 20 U daily, until death.

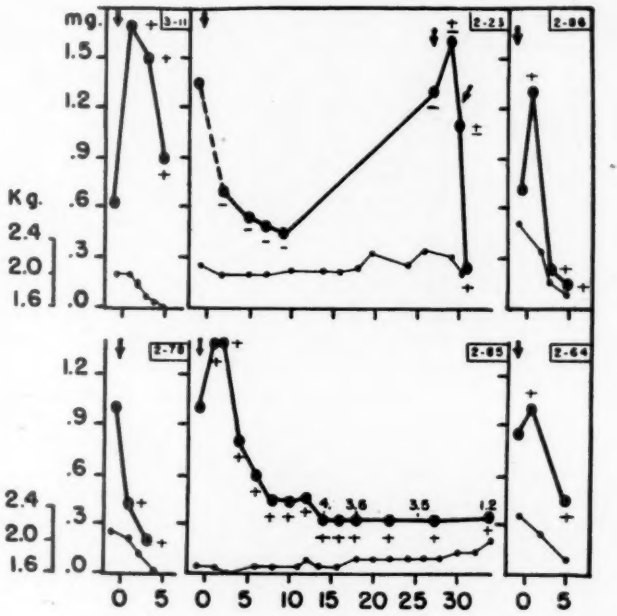


FIG. 1. — Six males: *Ordinates: thick lines: 17-ketosteroids, mg/24 hours; discontinuous line: no determination during the first 24 hours in animal 2/23 (anuria); thin lines: weight in kg. Abscissae: time in days. Other signs: positive Fehling; negative Fehling; arrow: alloxan injection. Figures above the 17-ketosteroids curve indicate glycemia in g per l.*

*Details referring to the six males: 3/11: Alloxantine, 150 mg per kg. Died the 5th day after injection. 2/23: Alloxan, first injection 200 mg per kg; second injection 200 mg per kg on the 28th day; third injection 300 mg per kg 3 days later. Diabetes appeared only after the third injection but the animal died the day afterwards. 2/86: Alloxan, 200 mg per kg. Died 4 days after the injection. 2/78: Alloxan, 200 mg per kg. Died 3 days after alloxan. 2/85: Alloxan, first injection 200 mg per kg; second injection 200 mg per kg on the 5th day. The observation ran up to the 34th day after the first injection of alloxan. 2/64: Alloxan, 200 mg per kg. Died 5 days afterwards.*

## RESULTS

The amount of 17-KS in normal males varied from 1.4 to 0.55 mg in 24 hours, with an average of 0.931 mg ± 0.108 (table I). In normal females the values ranged from 0.7 to 0.45 mg in 24 hours, with an average of 0.526 ± 0.026 mg. In serial determinations performed on the same animal during 40 days the amount of 17-KS eliminated by male or female in 24 hours was quite constant. Elimination of 17-KS was mar-

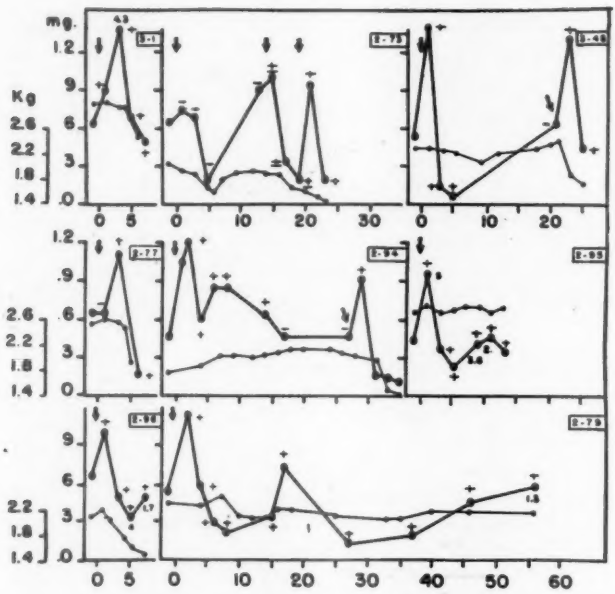


FIG. 2. — Eight females. *General specifications as in fig. 1.*

*Details referring to the eight females: 3/1: Alloxan, 100 mg per kg. Delivery 5 days after injection; litter died shortly after birth. After the 3rd day protamine-zinc insulin was injected, 20 U daily up to the death on the 8th day. 2/73: Alloxan, first injection 200 mg per kg; second and third injections of 200 mg per kg 14 and 20 days later. Died 4 days after the third injection. 3/48: Alloxan, first injection 150 mg per kg; second injection 200 mg per kg on the 22nd day. Died 2 days after the second injection. Transitory diabetes after the first injection. 2/77: Alloxan 200 mg per kg; 20 U of protamine-zinc insulin (PZI) were injected on the 4th day. Died 6 days after the alloxan injection. 2/94: Alloxan, first injection 100 mg per kg; second injection 200 mg per kg on the 28th day. Died 6 days after the second injection. Transitory diabetes after the first injection. 2/95: Alloxan 200 mg per kg. After 3rd day, PZI was injected 20 U daily. Died 14 days after the injection of alloxan. 2/96: Alloxan, 200 mg per kg; 50 U of PZI injected on the 5th day. Died 7 days after injection of Alloxan. 2/79: Alloxan, 100 mg per kg. Delivery 9 days later; all 5 young died next day. Diabetes lasted 2 months; when normal condition was reestablished excretion of 17-KS increased.*

TABLE I

*Urinary 17-hetosteroids in normal rabbits, during 40 days. All figures in mg per 24 hours.*

| Males | Prot. | Days | | | |
|---|---|---|---|---|---|
| | | 0-10 | 11-20 | 21-30 | 31-40 |
| 1 | 2/23 | 1.4 | | 1.3 | 1.4 |
| 2 | 2/78 | 1.1 | 1.0 | | |
| 3 | 2/85 | 1.0 | 0.950 | 1.0 | |
| 4 | 2/64 | 0.950 | 0.850 | | 0.750 |
| 5 | 2/86 | 0.700 | 0.700 | 0.750 | |
| 6 | 3/11 | 0.650 | | | |
| | | 0.700 | | | |
| | | 0.550 | | | |
| Average: 0.931 ± 0.108 | | | | | |
| **Females** | **Prot.** | **Days** | | | |
| | | 0-10 | 11-20 | 21-30 | 31-40 |
| 7 | 2/96 | 0.650 | 0.700 | 0.650 | |
| 8 | 2/73 | 0.650 | 0.700 | 0.600 | |
| 9 | 3/1 | 0.650 | | 0.650 | |
| 10 | 3/48 | 0.650 | 0.450 | | 0.650 |
| 11 | 2/77 | 0.650 | 0.650 | | |
| 12 | 2/79 | 0.650 | 0.550 | | 0.550 |
| 13 | 2/94 | 0.500 | 0.575 | 0.525 | 0.460 |
| 14 | 2/95 | 0.460 | | 0.475 | 0.450 |
| Average: 0.576 ± 0.026 | | | | | |

kedly altered in animals treated with quantities of alloxan sufficient to induce diabetes. The first effect is a considerable increase in the excretion of 17-KS, 24 to 48 hours after injection; the degree of the increase bears no relation to the amount of alloxan administered (figs. 1 and 2).

Usually after the aloxan-induced increase of urinary 17-KS a sharp drop followed. The latter was maintained while diabetes was present. All animals that died of diabetes, showed at this moment a very low level of 17-KS. On the contrary, in those rabbits that presented only a mild and transitory diabetes urinary 17-KS returned to normal values as soon as blood sugar diminished (2/79) or glycosuria disappeared (2/94 and 3/48). Sometimes the return to normal values was not complete as in rabbit (2/85) which presented a long though mild diabetes and showed

—after the initial increase— very low values of 17-KS; the latter persisted even after glycemia decreased from 4.5 to 1.2 g per l.

A decrease of urinary 17-KS took place even in those animals that were not rendered diabetic by alloxan (2/73 2/23); but in these cases the decrease was only of short duration and normal values reappeared soon. Also, after reinjection of alloxan, urinary 17-KS decreased following the usual initial increase.

The only animal that was injected with alloxantine (3/11) presented diabetes the day after injection. The initial increase of urinary 17-KS was, as with alloxan, followed by a decrease. The animal died with 17-KS values a little above normal.

Diabetes was more severe in males than in females.

Some details referring to individual animals will be especially mentioned. Animals 2/23 and 2/73 were not rendered diabetic by the first injection of alloxan; glycosuria appeared only after the third injection. At this moment there was a marked initial increase of 17-KS elimination in 2/73. On the contrary, in animal 2/23 which had received a larger dose of alloxan (300 mg per kg) and died the next day, no initial increase was produced and values of 17-KS were very low. The initial increase was lacking also in animal 2/78 which died the day following the injection of alloxan; glycosuria and hyperglycemia were very pronounced and urinary 17-KS very low. We shall return to these interesting divergencies when discussing our results.

Rabbits 2/94 and 2/97 showed, during the course of the disease, a spontaneous second though transient increase of urinary 17-KS, probably due to adrenal discharges related to the reestablishment of a normal steroid balance.

Two of the four diabetic animals treated with insulin (3/1 and 2/77) did not respond favorably and continued losing weight; they died with very low values of 17-KS. The remaining two (2/95 and 2/96) also died, though insulin had caused a considerable decrease of blood sugar. In both cases a slight increase of urinary 17-KS was observed prior to death.

Rabbits 3/1 and 2/79 gave birth to litters during the course of the observation.

## Discussion

The greater deviation of the mean of urinary 17-KS in our normal males compared to normal females may be explained by the influence of the testicle which may vary from one individual to another. Kimmeldorf (1948) found in normal male rabbits 1.12 mg $\pm$ 0.24 in 24 hours, whereas Koenig (1948) obtained 0.73 mg $\pm$ 0.04 in normal females. Results reported by Davis *et al.* (1949) are not coincident with the former ones; they found 2 mg of urinary 17-KS in 24 hours in males and even higher values in females. These divergencies are probably due to the different techniques employed.

As already insisted upon, there is in alloxan diabetic rabbits a considerable increase of urinary 17-KS in the first 24 or 48 hours after injection of the diabetogenic drug. After this initial increase the values drop under the normal level and remain low during all the time that diabetes persists, to rise slowly towards normal as soon as glycemia becomes normal and glycosuria disappears. This biphasic curve of 17-KS excretion after alloxan can be compared to what takes place in the alarm reaction as described by Selye (1950) and as corroborated by clinical observations. Selye has stated that alloxan intoxication causes an alarm reaction which becomes apparent by changes in the blood sugar (Jacobs, 1937). This effect —hyperglycemia of short duration followed by a

more prolonged hypoglycemia— is independent of the pancreas and is accompanied by other symptoms of the alarm reaction. According to Forbes *et al.* (1947) urinary 17-KS in man are considerably increased after the first or second day of exposure to stress. Afterwards, these values are greatly diminished reaching levels well below normal. In accord with the concept of Selye the initial increase of urinary 17-KS as found in our rabbits in which diabetes is induced by alloxan, may be interpreted as the phase of counter-shock of the general adaptation syndrome. The fall of urinary 17-KS may be due to the animal having reached the stage of resistance.

The work of Martínez and Orías (1945) is of considerable interest in our context. They found that toxic phenomena as induced by alloxan are more pronounced in previously adrenalectomized rats than in normal ones; adrenalectomized animals died within the first two or five hours with marked hypoglycemia. The results of Martínez and Orías might suggest that adrenalectomized animals are not able to respond with the counter-shock and therefore die during the stage of shock, which expresses itself by a very low glycemia. As shown by Martínez and Orías the shock may be prevented by the administration of adreno-cortical extracts.

On the other hand, the adrenals in alloxan-diabetic rats are larger than normally; histochemically a marked loss of substances soluble in acetone has been stated which was especially pronounced in the zona *fasciculata* and *reticularis;* these changes might be related to cortical stimulation, most probably by insulin, adrenaline or corticotropic hormone liberated by alloxan (Eränkö, 1951).

Again, in the work of de Majo (1947) with aloxan-diabetic rats there was in the beginning, seemingly, a strong stimulation of adrenal activity as evidenced by a decrease of adrenaline, cholesterol and ascorbic acid in the adrenals. Yet, no decrease of these substances took place when the animal was not rendered diabetic, i.e. when stimulation of the adrenals was supposedly absent. Something similar might have occurred in those of our animals that could not be rendered diabetic and did not respond with the initial increase of urinary 17-KS.

From the above consideration it seems reasonable to assume that in the experimentes reported here and in those of Majo the stimuli of the adrenals is not the alloxan alone, but alloxan and the diabetes induced by this drug.

In our opinion the whole evolution course of excretion of 17 - KS in rabbits with alloxan diabetes —diminished in those animals that were rendered diabetic, again normalized after transitory diabetes, or improved when insulin was effective— exteriorizes the changeable functional condition of the adrenals during the development of those general metabolic alterations which are produced in experimental diabetes. Clinical observations are in favor of our concept; in children with diabetes there is, at the beginning, a high level of urinary 17 - KS which in the course of years notably decreases (White, 1948).

It might be of interest to discuss our concept in the light of some other experimental and clinical findings. When rats were adrenalectomized during the evolution of an alloxan diabetes at 10 days after injec-

tion diabetes considerably improved (Janes and Friedgood, 1945). We have already mentioned that the same has been seen in diabetic patients with Addison's disease (Bloomfield, 1939). All these findings can be explained on the tentative assumption that diabetic symptoms depend partly on stimulation by gluco-corticoids. But on the other hand certain of our findings suggest that the functional condition of the adrenals is in alloxan diabetes more complex. We have seen that while the diabetic metabolic dysfunction is present, elimination of 17 - KS is very low; this effect tends to disappear when diabetes is of short duration only; also excretion of 17 - KS rises somewhat again when glycemia becomes normalized by insulin. All this would indicate that the adrenals are in hypofunction during alloxan diabetes. However it seems important to keep in mind that this hypofunction is most probably only a partial one, that is to say, that it refers only to certain corticoids and not to others. Thus, for instance there is in some endocrine disorders a dissociation of the various adrenal functions related to the production or liberation of corticoids; in Cushing's disease elimination of gluco-corticoids may be considerably increased while urinary 17 - KS remains normal (Venning and Browne, 1947). These observations, both experimental and clinical suggest that low urinary values of 17 - KS are not always an indication of a total hypofunction of the adrenals. One might speculate that in experimental diabetes and in diabetes in man there is an increased production or liberation of gluco-corticoids whereas that of testoids, or other corticoids, is decreased.

## Summary

Excretion of 17 - KS by the urine was in 6 normal male rabbits of $0.931 \pm 0.108$ mg in 24 hours. Excretion in 8 normal female rabbits was of $0.596 \pm 0.026$ mg in 24 hours.

Thirteen animals were injected with alloxan and one animal was given alloxantine. Those animals that were rendered diabetic presented a sharp rise in urinary 17 - KS during the first 24 to 48 hours after injection. This increase was followed by a sudden decrease which was maintained while the metabolic diabetic alteration was present.

During the course of a mild transitory diabetes the values of urinary 17 - KS tended to become normal as soon as the metabolic diabetic disturbance was over. Insulin, when improving diabetic symptoms, also caused an increase of urinary 17 - KS.

The evolutional course in the excretion of 17 - KS in rabbits under the influence of alloxan, is discussed from the point of view of Selye's concept of the alarm reaction and the general adaptation syndrome.

The various alterations in the elimination of 17 - KS indicate the important rôle played by adreno-cortical function in the initiation and development of alloxan diabetes.

## Bibliography

(1) Bloomfield: *Bull. Johns Hopkins Hosp.*, 1939, *65*, 456.
(2) Davis, C. T., Slater, C. R., Krischesky, B.: *Endocrinology*, 1949, *44*, 83.

(3) ENGSTROM, W. W., MASON, H. L.: *Endocrinology*, 1943, *33*, 229.
(4) ERÄNKÖ, O.: *Acta Endocrinologica*, 1951, *6*, 97.
(5) FORBES, A. P., DONALDSON, E. C., REIFENSTEIN, C., ALBRIGHT, F.: *J. clin. Endocrinol.*, 1947, *7*, 264.
(6) HAMBURGER, C.: *Acta Endocrinologica*, 1948, *1*, 19.
(7) JACOBS, H. R.: *Proc. Soc. exp. Biol., N. Y.*, 1937, *37*, 407.
(8) JANES, R. G., FRIEDGOOD, C. E.: *Endocrinology*, 1945, *36*, 62.
(9) KÖNNIG, J. DE, KRISCHESKY, B., GLASS, S. J.: *Proc. Soc. exp Biol.*, N. Y., 1948, *68*, 320.
(10) LONG, C. N. H.: *Harvey Lectures*, 1937, *32*, 194.
(11) MAJO, S. DE: *Rev. Soc. Argent. Biol.*, 1947, *23*, 46.
(12) MARTÍNEZ, C., ORÍAS, O.: *Rev. Soc. Argent. Biol.*, 1945, *21*, 290.
(13) SELYE, H.: *Textbook of Endocrinology*. Acta, Inc., Montreal, 1949.
(14) SELYE, H.: *Stress*. Acta, Inc., Montreal, 1950.
(15) TALBOT, N. B., BERMAN, R. A., MAC LACHLAN, E. A.: *J. biol. Chem.*, 1942, *143*, 211.
(16) VENNING, E. H., BROWNE, J. L.: *J. clin. Endocrinology*, 1947, *7*, 79.
(17) WHITE, P.: in JOSLIN, E. P., ROOT, E. H., WHITE, P., MARBLE, A., BAILEY, C. C.: *Treatment of diabetes mellitus*, Chapter XXVIII. Lea and Febinger, Philadelphia, 1948.
(18) ZAÑARTU, Y., ATRIA, A., ACEVEDO, H.: *Rev. Med., Chile*, 1950, *78*, 377.

# INFLUENCE OF VAGAL AND SYMPATHETIC DENERVATION ON THE EFFECTOR SYSTEMS OF THE MAMMALIAN HEART

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## INTRODUCTION

IN A previous work (8) from this laboratory it has been shown that the intracoronary injection of acetylcholine in the atropinized, isolated mammalian heart has a stimulating effect which resembles very closely the action of epinephrine, since it increases both the amplitude and frequency of atrial and ventricular contractions. The cardiostimulating effect of acetylcholine is concurrent with the release of an epinephrine-like substance, as shown by the fact that the cardiac perfusates collected during the periods of stimulation, exert an epinephrine-like action when estimated on various biological preparations (frog's hypodynamic heart, rectal cecum of the fowl, small intestine of the rabbit). It has been further demonstrated that the cardiostimulating effect of acetylcholine is abolished by nicotine and by sympatholitic agents like ergotamine. On the basis of these facts, the hypothesis has been advanced that in the heart itself and its proximity there are structures which under the action of acetylcholine release a substance pharmacologically similar to epinephrine. It seemed also reasonable to assume that these intra and juxtacardiac adrenergic structures are ganglion cells since, as already mentioned, the stimulating action of acetylcholine is suppressed by nicotine.

Mc Dowall (12) is inclined to admit that the cardiostimulating action of acetylcholine is a direct one, in view of his finding that it is not suppressed by nicotine; a tentative explanation for these results could be that in his experiments nicotinization was perhaps insufficient. A blocking action of ganglionic depressants on the cardiostimulating effect of acetylcholine has been confirmed by McNamara *et al.* (13), who have further shown that calcium ions reverse this block.

One of us ([14]) has later shown that stimulation of the cervical vagus in the atropinized isolated heart, induces cardiostimulation, very similar to that obtained with acetylcholine, this effect being, also, concomitant with the liberation of an epinephrine-like substance. These results were interpreted as indicating the possibility of intra and juxtacardiac adrenergic ganglion cells being under the control of vagal preganglionics. In other words, it would be an instance of parasympathetic postganglionic neurones acting on the heart effectors (cardiac muscle and cardiovector system) by the mediation of a transmitter substance characteristic of sympathetic postganglionics. This hypothesis would involve an exception to the general pattern of functional integration of the parasympathetic system; but it may be recalled, that another "anomaly" has since long been described in connection with the sympathetic system: the cholinergic innervation of the sweat glands.

The possibility that the cardiostimulation obtained by excitation of the cervical vagus might be due to the presence of aberrant sympathetic fibres, was ruled out by experiments in which the cervical sympathetic chains were excised bilaterally some 15 days before. In all these preparations the cardiostimulating effects of the vagus were shown to persist unchanged ([14]).

According to the "Law of Denervation" ([5]), if there were in the mammalian heart adrenergic neurones connected to vagal preganglionics, degeneration of the latter would lead to sensitization of the adrenergic neurones to acetylcholine; furthermore, a sensitization of both the heart muscle and pace-maker to epinephrine could also be expected. Degeneration of the sympathetic postganglionics, on the other hand, would sensitize the heart, not only to epinephrine, but also to the stimulating action of acetylcholine.

In the present paper, experiments are reported concerning the response of the cardiac effectors to neurotransmitter substances, after degeneration of the vagal or sympathetic cardiac fibres.

## METHODS

Cats of 2 — 3 kg body weight, anesthetized with Dial (0.07 — 0.08 k/kg; intraperitoneally) were used.

The experiments were carried out on the isolated vagus-heart preparation. The heart was perfused with Tyrode's solution as described elsewhere ([8]); both vagi were dissected in their intrathoracic course and cut cephalad to the heart; the nerves were placed on shielded, bipolar, silver wire electrodes and stimulated with sinusoidal 50 cycles per second AC, the voltage being regulated by means of potentiometers.

Ventricular contractions were recorded on smoked paper, with an isotonic lever attached to the apex of the heart.

Acetylcholine (clorhydrate), epinephrine (clorhydrate) and nicotine (bitartrate) were injected into the aortic cannula close to the heart, always in the same volume (0.1 $cm^3$) and with the same speed. In order to suppress the cardiodepressor effects of acetylcholine, the preparations were "atropinized" by adding Bellafoline "Sandoz" *, in concentrations from 0.025 to 0.1 mg per liter of Tyrode solution.

Degeneration of the cardiac sympathetic fibres was obtained by removing on

* We are grateful to "Oficina Científica Sandoz", Chile, for the generous supply of Bellafoline.

both sides the stellate ganglia, and the thoracic lateral chains, following the technique described by Cannon (4).

The cardiac vagus fibres were allowed to degenerate by vagotomy performed according to the technique recommended by Hodes (7); the right vagus was severed within the thorax, immediately below the emergence of the recurrent nerve; section of the left vagus was made in the lower part of its cervical course. By this procedure, the risk of damaging the sympathetic innervation of the heart is minimized.

These preparations will be referred to as "sympathectomized" and "vagotomized" hearts respectively.

The experiments on the isolated vagus-heart preparations were performed between 12 and 16 days after denervation, in order to allow a complete degeneration of the respective nerve fibres.

## RESULTS

### I) Effects of acetylcholine.

*A) Normally innervated hearts.*

In experiments performed on 20 non-denervated hearts, it was shown, as previously described (8), that acetylcholine injected in doses of 25-50 µg induced a biphasic effect, characterized by an initial period of decreased ventricular activity, followed by a period of stimulation during which the rate and amplitude of the contractions were markedly increased.

Smaller doses of acetylcholine induced only slowing of the heart contractions, the threshold doses for this effect being 0.46 ± 0.03 ug. Even smaller doses (0.1 and 0.3 µg) had no chronotropic effect, but induced a slight increase in amplitude of the ventricular contractions.

After suppressing the cardiodepressor effect by atropinization (Bellafoline), the response of the heart to acetylcholine was characterized by an increase of the amplitude and frequency of the contractions (nicotinic action of acetylcholine 8). The minimal doses of acetylcholine necessary to increase in 5 - 10 % the amplitude of ventricular contractions were of 20.5 ± 2.52 µg. When the concentration of acetylcholine was augmented, the positive inotropic effects were progressively increased; maximal effects were obtained, generally, with 100 µg, the amplitude increasing an average of 130 %.

The threshold doses of acetylcholine capable of eliciting an accelerating effect (increase in frequency of 5 - 10 %) were remarkably variable, ranging in the different preparations from 5 - 100 µg. With overthreshold doses the effect on the pace-maker was proportionally increased, maximal rate increments ranging from 30 - 65 %.

*B) "Vagotomized" hearts.*

In 12 experiments, it was observed that both the depressor and stimulating effects of acetylcholine persisted. In these hearts, slowing of the ventricular rate was obtained with threshold doses of acetylcholine ranging from 0.1 to 0.05 µg, which were markedly lower than those found in normally innervated and "sympathectomized" hearts.

After atropinization, the increase in amplitude of the ventricular contractions was produced with minimal doses of acetylcholine of 10.5 ± 1.0 µg, which were significantly lower than the threshold doses found in normally innervated hearts. Maximal stimulating effects were

obtained with doses as low as 50 μg, producing an average increase of 140 % in the amplitude of the contractions.

The threshold doses of acetylcholine capable of accelerating ventricular contractions and the maximal chronotropic responses, were of the same order of magnitude as in normally innervated hearts.

*C) "Sympathectomized" hearts.*

In 11 experiments, it was found that both the depressor and stimulating effects of acetylcholine were present. The threshold doses of acetylcholine inducing slowing of ventricular contractions, were similar to those found in normally innervated preparations.

In the atropinized preparations, positive inotropic effects were elicited with mean threshold doses of 12.2 ± 1.55 μg of acetylcholine. Maximal effects were obtained with average doses of 100 μg, which induced only a slight increase (average 60 %) in the amplitude of the ventricular contractions, which was markedly smaller than that observed in the normally innervated and "vagotomized" hearts.

These preparations differ very markedly from the normally innervated and "vagotomized" hearts, with regard to the reaction of the pacemaker to the stimulating action of acetylcholine. Only in one, —out of the 11 hearts of this group—, a slight increase in rate was observed. In the remaining 10 preparations no increase in cardiac frequency was noticed, even with doses of acetylcholine as large as 100 μg.

**II) Effects of epinephrine on normally innervated, "vagotomized" and "sympathectomized" hearts.**

It was observed that epinephrine exerts a positive chrono and inotropic action on the hearts with intact innervation as well as in the denervated ones. The average threshold doses of epinephrine which increased the amplitude of ventricular contractions in the normally innervated hearts were found to be 0.025 ± 0.005 μg, in the "vagotomized" hearts 0.012 ± 0.004 μg, and in the "sympathectomized" hearts, 0.016 ± 0.006 μg. The threshold differences of the 3 groups are not statistically significant. The positive inotropic action of epinephrine on the ventricles was similar in the normally innervated and "vagotomized" preparations; in both groups, progressively increasing doses of epinephrine induced proportionally increasing responses; with average maximal doses of 0.5 μg the average increment in amplitude was 110 %.

In "sympathectomized" hearts, the positive inotropic effect of epinephrine was surprisingly small and there was no proportionality between the doses given and the magnitude of the ventricular reaction. Such preparations always showed the same slight response (an average 20 % increase in amplitude) irrespective of the doses of epinephrine given (from 0.05 to 0.5 μg). The duration of the cardiostimulating effect is, however, proportional to the concentrations of epinephrine injected.

The average minimal doses of epinephrine, which produced cardiac acceleration, were similar in the 3 groups of hearts. Overthreshold doses induced the same degree of acceleration in normally innervated and "vagotomized" hearts, while the increase in cardiac rate of "sympathecto-

mized" hearts, was more marked. Thus, in the first two groups, 0.05 and 0.5 μg of epinephrine augmented the frequency in an average of 25 and 50 %, while in the "sympathectomized" hearts the same doses increased the rate in 40 and 80 %, respectively.

**III) Effects of vagus stimulation in normally innervated and "sympathectomized" hearts.**

In 18 experiments carried out on atropinized hearts with degenerated sympathetic innervation, stimulation of the vagi was followed by an increased amplitude and frequency of the ventricular contractions.

**IV) Effect of nicotine on normally innervated, "vagotomized" and "sympathectomized" hearts.**

Nicotine in doses of 5 and 20 μg regularly induced in the atropinized hearts (7 normally innervated, 6 vagotomized and 5 sympathectomized) the well known stimulating effect, i.e., increased amplitude and frequency of ventricular contractions. In normally innervated and "vagotomized" hearts the magnitude of the responses to this drug was similar; in "sympathectomized" preparations, however, it exerts a rather slight effect on the amplitude of the contractions, a phenomenon which has been already mentioned as characteristic of the responses of the "sympathectomized" hearts to epinephrine and acetylcholine.

## Discussion

The present work was primarily planned to see if the juxta and intracardiac adrenergic ganglia are modified in their sensitivity to acetylcholine as a consequence of decentralization. The results (see table I) show that significant sensitization of these ganglionic structures is induced by degeneration of both vagal and sympathetic cardiac nerves; thus, the threshold doses of acetylcholine necessary to increase the amplitude of the ventricular contractions are significantly lower in the denervated hearts.

In the hearts with degenerated vagal innervation, the results are according to expectation in view of the fact that, as shown by one of us (14), the adrenergic intracardiac ganglia are apparently under vagal control.

The fact that sympathetic denervation leads also to sensitization of the adrenergic ganglia, can be interpreted as indicating that these structures are controlled by impulses proceeding from sympathetic preganglionic fibres as well. It is generally admitted that the synaptic relay between the first and second sympathetic neurones occurs in the stellate and intrathoracic ganglia, the sympathetic fibres which innervate the heart being therefore all of the postganglionic type. However, the complexity of the cardiac plexus, in which vagal and sympathetic fibres are mingled, makes it impossible to differenciate histologically between the two groups of axons. In addition, the existence of different kinds of ganglion cells in the myocardium, epicardium and adjacent connective tissue has made it most difficult to get a clear picture of the connections of these ganglia with the extrinsic heart nerves. A. Kuntz (11) writes:

"...in view of the intimate relationship of the cardiac ganglia to the mixed fibre bundles in the cardiac plexus it has seemed to some investigators that both sympathetic and vagus fibres make synaptic connections in the cardiac ganglia...", and he adds that "...this view has not been confirmed by results of physiological experiments".

TABLE I

*Sensitivity and maximal response to acetylcholine and epinephrine in "vagotomized" and "sympathectomized" hearts, as compared with normally innervated hearts.*

| | | Acetylcholine | | Epinephrine | |
|---|---|---|---|---|---|
| | | Inotropic action | Chronotropic action | Inotropic action | Chronotropic action |
| Sensitivity | Vagotomized | Increased | Unchanged | Unchanged | Unchanged |
| | Sympathect. | Increased | No response | Unchanged | Unchanged |
| Maximal response | Vagotomized | Unchanged | Unchanged | Unchanged | Unchanged |
| | Sympathect. | Decreased | No response | Decreased | Increased |

There are certain embryological considerations which support the hypothesis of the existence of intra and juxtacardiac ganglia connected to preganglionic sympathetic fibres. Thus, according to Arey (2) "...in addition to the primary ganglia of the paired sympathetic trunk... there are other more peripheral ones... belonging to the prevertebral plexus... still further distal are the terminal ganglia located near or even within the structures they innervate; this group includes the ciliary and cardiac ganglia..." According to Nonidez (15), it is rather easy to observe in the embryonic heart that, during the migration of the sympathetic neuroblastas, some of these do not stop in the ganglia but migrate more peripherally to form the intra and juxtacardiac ganglia.

Thus, from a morphological point of view, there is some evidence which favours the existence of interneuronal sympathetic synapsis in the heart itself or in the surrounding tissues.

Since in "vagotomized" hearts there appears to exist a sensitization of adrenergic ganglionic structures to acetylcholine, it seemed reasonable to expect in these hearts a more marked positive chronotropic action of this substance as well. The analysis of our records, however, has shown that there is no difference between "vagotomized" and normally innervated hearts concerning the responsiveness of the pace-maker to acetylcholine. In the "sympathectomized" preparations a rather puzzling phenomenon is observed: in these hearts acetylcholine does not modify the frequency of the contractions, even when given in high concentrations. This fact is difficult to harmonize with the hypothesis that acetylcholine acts through an adrenergic mechanism. It cannot be explained on the

basis of a diminished responsiveness of the pace-maker, since in these preparations the chronotropic action of epinephrine is increased.

The magnitude of the positive inotropic effect of acetylcholine is similar (see table) in normally innervated and "vagotomized" hearts (the maximal average increase in amplitude being of the same order in both groups). But in the sympathectomized preparations the ventricular response is significantly decreased, a fact indicating (see later) that the responsiveness of the myocardium to the mediator released by the adrenergic cardiac ganglia is reduced. In the sympathetically decentralized heart, therefore, the responsiveness to acetylcholine of both the pace-maker anh the myocardium is markedly diminished.

With respect to the action of epinephrine on the frequency and amplitude of the ventricular contractions, it is observed (see table) that the minimal effective amounts of this substance are of the same order of magnitude in the normally innervated and denervated hearts. Overthreshold doses induce proportionally increasing inotropic and chronotropic responses of the ventricles in the normally innervated and "vagotomized" preparations; but in the "sympathectomized" hearts the increase in amplitude of the ventricular contractions is much smaller and no augmentation of the effect is observed when high concentrations of this substance are injected, thus having the same characteristics already described in connection with the action of acetylcholine. Apparently, therefore, the sympathetic decentralization of the heart induces biochemical changes of the myocardium which reduce its capacity to increase the strength of the contractions under the action of epinephrine-like substances. Unfortunately, no experiments were performed in order to decide if these changes are specific for epinephrine or extend to other cardiostimulating agents. The reduced capacity of the ventricular muscle of sympathectomized hearts, to increase the amplitude of contractions under the action of epinephrine, is a constant phenomenon which —as far as we know— has not been described.

Our results are concordant with the observations of Burret ([3]), since the "responsiveness" of the pace-maker to epinephrine (see table) appears augmented in "sympathectomized" hearts, compared with the normally innervated ones. It is interesting to point out, however, that, as already mentioned, the "susceptibility" of the pace-maker is not modified by sympathectomy, as shown by the fact that the threshold doses of epinephrine with accelerating effect are similar in both groups of preparations.

As it might be expected according to the "Law of Denervation", in the "vagotomized" preparations the pace-maker appears sensitized to the depressing action of acetylcholine. Unpublished results from this laboratory, have shown that the amplitude of contraction of ventricles beating with an idioventricular rhythm is not depressed by acetylcholine. In view of this fact we refer only to the depressing action of acetylcholine on the rate of contractions.

The results mentioned in the Introduction, concerning preparations in which the cervical sympathetic had been allowed to degenerate, together with the present observations, that after degeneration of the cardiac sympathetic supply the stimulating action of the vagus persists, indicate

that the cardiostimulating fibres of the vagus are of medullary origin and not —as had been supposed— "aberrant fibres" of the sympathetic system enclosed in the vagus trunk.

The existence in the cardiac vagal system of second neurones of adrenergic nature would not represent a unique and extravagant phenomenon, since Ambache (1) has recently shown that in the isolated stomach and intestine the contractions induced by nicotine are reversed after atropinization. According to this author, the relaxation is produced through an adrenergic ganglionic mechanism controlled by the vagus, since it is abolished by hexamethonium and ephedrine.

The existence of vagus fibres connected to adrenergic heart ganglia may lead to a better understanding of the results of H. Kabat (10) and others (9) (16) (17), who observed that stimulation of the vagus or its intracranial roots, in atropinized dogs, produces heart acceleration even in those preparations in which the upper cervical cord was cut and the thoracic sympathetic chain severed. Kabat found, in agreement with Jourdan and Nowak (9) that in preparations (dog) in which the head is connected to the trunk by only the vagi, cerebral anemia induces bradycardia, this response being reversed after atropinization. Based on these results he formulates the hypothesis of the existence of adrenergic fibres in the vagus system.

Haney and Lindgren (6) observed that the intravenous injection of acetylcholine into non-anesthetized atropinized dogs, with completely denervated hearts, induces a marked tachycardia. In view of the short latency of the effect, the authors are inclined to admit a stimulating action of acetylcholine directly on the heart or on intracardiac adrenergic ganglia.

## SUMMARY

The effects of acetylcholine and epinephrine on the effector systems of hearts with intact innervation or with vagal or sympathetic degenerated innervation, are analyzed.

In hearts with *degenerated vagal nerves*, the negative chronotropic action of acetylcholine is increased. In the atropinized hearts the threshold for the positive inotropic action of acetylcholine appears significantly lowered, a fact that is interpreted as due to sensitization, by denervation of adrenergic juxta and intracardiac ganglia. The capacity of the ventricles to increase the amplitude of their contractions under the action of acetylcholine (activation of adrenergic ganglia) is comparable to that observed in normal hearts. There are no changes of the sensitivity nor of the responsiveness of the heart for the positive chronotropic action of acetylcholine. No changes are observed concerning the response of the heart to epinephrine.

*Degeneration of the cardiac sympathetic innervation* induces a significant lowering of the threshold for the positive inotropic action of acetylcholine. It is confirmed, for the isolated heart, that sympathetic denervation leads to an increased chronotropic effect of epinephrine, even though the threshold for this effect is not modified. Moreover, the positive chronotropic response of the heart to acetylcholine appears abo-

lished. In these hearts a reduced capacity of the ventricles to increase the strength of contraction under the action of epinephrine or following the activation (by acetylcholine or nicotine) of the adrenergic ganglionic structures, is regularly observed.

On the basis of the results obtained it is concluded that they give further support to the view that there exist vagal preganglionic fibres controling the activity of juxta and intracardiac adrenergic ganglia. The possibility is discussed of these ganglia being also under the control of sympathetic preganglionics.

## REFERENCES


(1) AMBACHE, N., EDWARDS, J.: *Brit. J. Pharm. Chemother.*, 1951, *6*, 311.
(2) AREY, L. B.: *Developmental Anatomy.* Philadelphia, W. B. Saunders, 1941, p. 470.
(3) BURRET, J. B.: *Am. J. Physiol.* 1940, *131*, 409.
(4) CANNON, W. B.: *Am. J. Physiol.*, 1926, *77*, 326.
(5) CANNON, W. B., ROSENBLUETH, A.: *The Supersensitivity of Denervated Structures. A Law of Denervation.* New York, The Macmillan Co., 1949.
(6) HANEY, H. F., LINDGREN, A. J.: *Am. J. Physiol.*, 1945, *145*, 177.
(7) HODES, R.: *Am. J. Physiol.*, 1939, *126*, 171.
(8) HOFFMANN, F., HOFFMANN, E. J., MIDDLETON, S., TALESNIK, J.: *Am. J. Physiol.*, 1945, *144*, 189.
(9) JOURDAN, F., NOWAK, S. J. G.: *Arch. Int. Pharmacol.*, 1936, *53*, 122.
(10) KABAT, H.: *Am. J. Physiol.*, 1940, *128*, 246.
(11) KUNTZ, A.: *The Autonomic Nervous System.* Philadelphia, 1934, p. 136.
(12) MCDOWALL, R. J. S.: *J. Physiol.*, 1946, *104*, 392.
(13) MCNAMARA, B., KROP, S., MCKAY, E. A.: *J. Pharmacol. Exp. Therap.*, 1948, *92*, 153.
(14) MIDDLETON, S., MIDDLETON, H. H., TOHÁ, J.: *Am. J. Physiol.*, 1949, *158*, 31.
(15) NONÍDEZ, J. F.: (personal communication), 1944.
(16) SCHIFF, M.: *Pflüger's Arch.*, 1878, *18*, 172.
(17) TULGAN, J.: *Am. J. Physiol.*, 1923, *65*, 174.

# EFFECT OF ACETYLCHOLINE, NORADRENALINE, ADRENALINE AND HISTAMINE ON ISOLATED ORGANS OF APLYSIA AND HOLOTHURIA

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THE EFFECTS of neurohormones and various drugs on heart and other organs of lower animals are of considerable interest not only in view of their possible relations to the autonomic nervous system but also with regard to the development in the animal series of specific receptor mechanisms in the reacting organs. A more detailed knowledge in this field might serve as a basis for a functional classification different from that generally applied in animal systematics.

A great number of data may be obtained from the reviews published by Bacq (1946), Frédéricq (1947), Prosser (1946) Hanström (1939), Burn (1950) and Prosser (1950).

Some experiments on the action of autonomic drugs on isolated organs from *Aplysia* and *Holothuria* from the Recife area are reported in the present paper.

## MATERIAL AND METHOD

The animals, *Aplysia dactylomela* and *Holothuria*, were caught on the day of the experiment and kept in sea water in the laboratory.

The following isolated organs were used for the tests: *Aplysia* heart, with and without adjacent ganglia, pharyngeal sac, esophagus, intestine and tunical muscle. *Holothuria:* water lungs, small intestine, longitudinal muscle.

The organs were prepared and suspended in a 20 ml bath containing sea water diluted with distilled water to 80 %, and airated with oxygen. All experiments were performed at room temperature (25° C).

The drugs used were acetylcholine "Roche", dl—noradrenaline "Winthrop", adrenaline "Parke, Davis & Co.", histamine "Roche", nicotine tartrate, atropine sulfate, and eserine.

I. *Aplysia*

a. *Heart.* — Some experiments were made on the heart freed from adjacent ganglia. On such preparations acetylcholine in doses of 20 $\mu$g and large (conc. $10^{-6}$) produced a slow contraction (fig. 1C). No spontaneous movements were observed in hearts without ganglia in this experiment. If however, the heart were prepared together with its ganglia, spontaneous beats were recorded in all cases, with a frequency

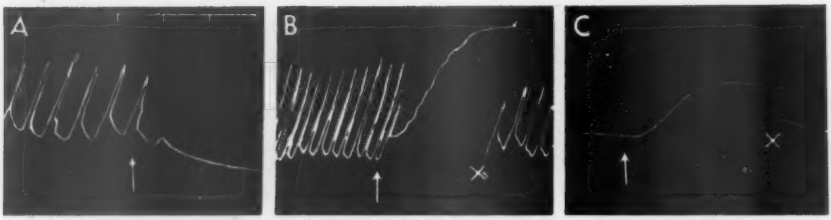


FIG. 1. — *Isolated heart, with ganglia, of* Aplysia dactylomela *in 80 per cent sea water. Volume of bath 20 ml, airated with oxygen, 25° C. A. 0.1 µg acetylcholine. B. 20 µg acetylcholine. C. (without extracardial ganglia) 20 µg acetylcholine. Time marks 20 s; x exchange of bath fluid.*

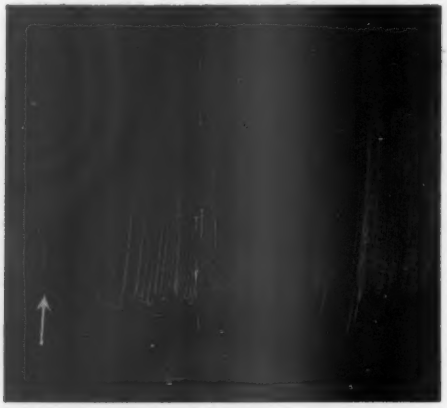

FIG. 2. — *Isolated heart, with ganglia, of* Aplysia dactylomela. *0.3 mg nicotine. Heart frequency increasing to 44 per m.*

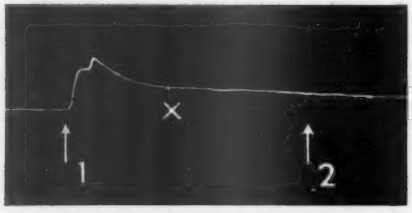


FIG. 3. — *Pharyngeal sac (papo) of* Aplysia dactylomela. *1: 100 µg dl-noradrenaline. 2: 50 µg l-adrenaline.*

of about 6—10 per minute. These preparations showed a high sensitivity to acetylcholine. Thus 0.02 $\mu$g acetylcholine ($10^{-9}$) (without eserine) diminished the frequency but did not stop the heart. Doses of 0.1 $\mu$g caused a complete inhibition of the beats and relaxation of the heart muscle (fig. 1A). A similar effect was obtained with doses of 0.2-5 $\mu$g ($10^{-8}$ — $2.5 \cdot 10^{-7}$). Doses of 20 $\mu$g ($10^{-6}$) produced a quick contraction followed by a slow sustained one (fig. 1C). In preparations which did not beat initially a dose of 20 $\mu$g acetylcholine usually was followed by rhythmic activity after change of bathing fluid and subsidence of the contraction.

Since it seemed that the inhibitory action was due to the presence of ganglia, the action of atropine was investigated. Concentrations of atropine up to $10^{-8}$ did not influence the inhibitory action of acetylcholine and also had no action on the stimulating effect of higher doses of acetylcholine. This seemed to exclude the possibility that acetylcholine produced its inhibitory effect by a muscarinic action and indicated rather a nicotinic action on the ganglionic cells. This assumption was also supported by the results with nicotine, which in a dose of 0.3 mg, caused inhibition and standstill, followed by a strong stimulation and increase of the frequency (fig. 2).

As might be expected the addition of eserine to the bath increased the effect of

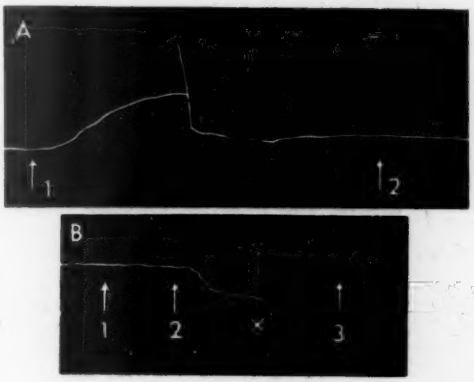


FIG. 4. — *A: Isolated water-lung of* Holothuria. *Sea water. 1: 50 $\mu$g histamine di-HCl. 2: 100 $\mu$g acetylcholine. B: 1: 50 $\mu$g acetylcholine. 2: 20 $\mu$g dl-noradrenaline. 3: 10 $\mu$g l-adrenaline.*

acetylcholine. After eserinization it was also noted that spontaneous rhythmical contractions ceased, indicating a disturbance of the normal production of impulses initiating the heart beats.

The effect of noradrenaline and adrenaline was also tested in a number of cases. Even with such high doses as 50 $\mu$g adrenaline and 100 $\mu$g ($2 \cdot 10^{-5}$) dl-noradrenaline no effect could be detected in the majority of cases. In one case it seemed that those doses produced relaxation and standstill of the heart followed by irregular activity.

b. *Pharyngeal sac* (papo). — Spontaneous contractions of low frequency were noted in some cases. Contractions of the pharyngeal sac were obtained with acetylcholine in doses of 20 $\mu$g and higher (conc. $10^{-6}$).

dl-Noradrenaline regularly caused contractions in doses of 100 $\mu$g and higher, whereas adrenaline in corresponding doses had no effect (fig. 3).

Atropine in a concentration of $10^{-6}$ did not inhibit the effect of acetylcholine, which therefore appeared to be of the nicotinic type. Nicotine in a dose of 0.5 mg also produced contraction.

Histamine (50 $\mu$g) had no visible effect.

c. *Oesophagus.* — The oesophagus responded to acetylcholine in doses of 1 $\mu$g and higher by contraction. Even on this preparation dl-noradrenaline in doses of 40 $\mu$g and higher induced a contraction whereas adrenaline in amounts up to 100 $\mu$g had no effect.

d. *Tunic muscle.* — In doses of 20 $\mu$g and higher acetylcholine caused a slow contraction.

II. *Holothuria*

a. *Water-lungs.* — No response was obtained with acetylcholine in amounts up to 100 $\mu$g. On this preparation histamine was active, however, causing a slow contraction in doses of 50 $\mu$g (fig. 4A).

The response to dl-noradrenaline and adrenaline was notable, in that both substances caused a relaxation in doses of 3 $\mu$g and higher (fig. 4B).

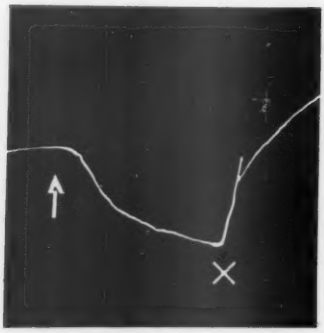

FIG. 5. — *Isolated longitudinal muscle of* Holothuria, *50 $\mu$g histamine.*

b. *Intestine.* — On the oral portion of the intestine acetylcholine induced a slow contraction in doses of 20 $\mu$g, as did nicotine (50 $\mu$g or more).

No effect was observed with dl-noradrenaline or adrenaline.

c. *Longitudinal muscle.* — This preparation was very sensitive to acetylcholine as seen by the fact that 0.2 $\mu$g caused a contraction in the absence of eserine. In higher doses the effect was stronger and more lasting.

No effect was observed with adrenaline and dl-noradrenaline, but histamine regularly caused a relaxation (fig. 5).

## DISCUSSION

The inability of atropine to block the action of acetylcholine in *Aplysia limacina* was first observed by Heymans (1924) and has been repeatedly confirmed for other mollusc hearts by other authors. The same applies to the nervous stimulation of the heart of the mollusc *Venus* (Prosser 1940).

Our results have confirmed that the inhibitory action of small doses of acetylcholine on the *Aplysia* heart is not blocked by atropine. The action can therefore not be muscarinic in nature.

Our experiments do not show decisively whether the rapid inhibitory action of acetylcholine is via the ganglia or is a direct effect on the muscle fibre, but since larger doses of acetylcholine have a slow stimulating action, seemingly independent of the ganglia, one might assume that the former action is on the ganglia. The effect of acetylcholine in

our experiments is rather similar to that obtained with excess potassium in the heart of *Aplysia limacina* by Heymans (1924). The contracture of the molluscan heart in response to acetylcholine was earlier observed by Jullian and Vincent (1938).

It is not known whether the adrenergic mediators, noradrenaline and possibly adrenaline, occur in the *Aplysia* heart. At any rate our experiments have not given any indication that the heart of *Aplysia* possesses receptors capable of reacting to noradrenaline or adrenaline in moderate doses. It is of interest that Hykes (1930) found that the stimulating effect of adrenaline on the *Pterotracheo* largely disappeared after removal of the subesophageal ganglia. On *Helix pomatia* Gautrelet and Halpern (1935) and Boyer (1926) found no stimulating effect of adrenaline.

On the other hand the smooth muscle of the pharyngeal muscular sac and the esophagus show some capacity to react to noradrenaline in doses where adrenaline is without effect, although the sensivity is low.

More surprising perhaps is the relaxation of the water lung of *Holothuria* in response to small doses of noradrenaline and adrenaline, acetylcholine in this case being ineffective. This organ was also sensitive to histamine, like the longitudinal muscle of the *Holothuria*, although the latter organ in contrast to the former, responded to histamine with relaxation.

Our experiments have given some further indication of the variability in response of various organs of lower animals to autonomic drugs. A further systematic study of this kind seems desirable in order to obtain a more detailed picture of the receptor systems of striated and smooth muscle for autonomic drugs.

## SUMMARY

The isolated heart of the mollusc *Aplysia dactylomela* was found to beat rhythmically in the presence of the heart ganglia. Acetylcholine inhibited the activity in low concentrations ($10^{-9}$). This action was not blocked by atropine (in confirmation of Heymans (1924) and others). Larger concentrations of acetylcholine ($10^{-6}$) or more) caused slow sustained contractions in the non-beating heart.

Moderate doses of noradrenaline and adrenaline had no effect on the isolated *Aplysia* heart, but noradrenaline unlike adrenaline in similar doses stimulated the pharyngeal sac of *Aplysia*.

In *Holothuria* histamine caused a contraction and noradrenaline, like adrenaline, a relaxation of the water lung, whereas acetylcholine had no effect.

The longitudinal muscle of *Holothuria* contracted in response to small doses of acetylcholine and relaxed with histamine.

## REFERENCES

BACQ, Z. M.: L'acétylcholine et l'adrénaline chez les invertébrés. *Biol. Rev. Cambridge Phil. Soc.*, 1947, *22*, 73.

BOYER, P.: Action de l'adrénaline et de la spartéine sur le cœur de l'Escargot. *C. R. Soc. Biol., Paris*, 1926, *95*, 1244.

BURN, J. H.: Relation of Motor and Inhibitor Effects of Local Hormones. *Physiol. Rev.*, 1950, *30*, 177.

FREDERICQ, H.: Les nerfs cardio-régulateurs des invertébrés et la théorie des médiateurs chimiques. *Biol. Rev.*, 1947, *22*, 297.

GAUTRELET, J., HALPERN, N.: Antagonisme du curare, du cyanure d'éthyle et de l'hémolymphe vis-à-vis de l'action inhibitrice de l'acetylcholine sur le coeur *d'Helix pomatia*. Influence de l'ésérine. *C. R. Soc. Biol., Paris*, 1935 *118*, 412.

HANSTRÖM, B.: *Hormones in invertebrates*. Oxford, Clarendon Press, 1939.

HEYMANS, C.: Influence des ions et de quelques substances pharmacodynamiques sur le coeur d'*Aplysia limacina*. *Arch. int. Pharmacodyn.*, 1923/24, *28*, 337.

HYKES, Q. V.: L'adrénaline et le coeur des Mollusques. *C. R. Soc. Biol., Paris*, 1930, *103*, 360.

JULLIEN, A., VINCENT, D.: Sur l'action de l'acétylcholine sur le coeur des Mollusques. L'antagonisme curare-acétylcholine. *C. R. Acad. Sci.*, 1938, *206*, 209.

PROSSER, C. L.: Acetylcholine and nervous inhibition in the heart of *Venus mercenaria*. *Biol. Bull. Woods Hole*, 1940, *128*, 92.

PROSSER, C. L.: The physiology of nervous system of invertebrate animals. *Physiol. Rev.*, 1946, *26*, 337.

PROSSER, C. L., BISHOP, D. W., BROWN, F. A., JAHN, T. L., WULF, V. J.: *Comparative Animal Physiology*. London, W. B. Saunders Company, 1950.

# EFFECT OF AROMATIC AMINES ON NEUROMUSCULAR FUNCTION (*)

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AS IT IS well known, the effects of a great number of aromatic amines on the smooth muscles of blood vessels and bronchi have been extensively reported. Nevertheless, only a few of them have been investigated on neuromuscular function, adrenaline being especially studied ([3, 4, 5, 6, 8, 10, 18, 19 and 20]).

The object of the present work was to study the effects of some aromatic amines on the neuromuscular preparation in order to learn whether certain relations between chemical structure and pharmacological activity could be established. To explore this point we investigated the effect of these amines, on single muscular twitch, on the neuromuscular block produced by C-10 and d-tubocurarine and on the effects of small doses of neostigmine and decamethonium. Special attention was directed toward adrenaline considering that it is the aromatic amine with the best known neuromuscular effects.

## METHODS

Unselected adult cats weighing from 1 450 g to 4 300 g were used. They were anaesthetized with 1 $cm^3$/Kg of a solution of pentobarbital-urethane given intraperitoneally (25 % urethane in 3.3 % sodium pentobarbital). Some animals were anaesthetized intravenously with chloralose (80 mg per Kg).

Most experiments were performed on the *quadriceps femoris*, but the *tibialis anterior* was also employed. The femur and tibia were fixed by means of drills. The distal tendons of the muscles were dissected free and attached to the short arm of a writing lever pulling against elastic bands. Both muscles were stimulated through the nerve by condenser discharges with pulse durations from 0.10 to 0.13 ms, which in turn were controlled by vacuum tubes. The nerve was crushed proximally. Maximal stimuli were applied. The stimulating electrodes were made of silver wires insulated with rubber. A cannula was inserted in the trachea so that artificial respiration could be used when necessary. Action potentials were led off from the belly of the muscle by means of recording electrodes consisting of two steel needles.

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The potential transient fed a Grass pre-amplifier, which in turn was connected to a Cossor double beam oscillograph (for details of this preparation, see [14]). The trace was then photographed. Recording of end-plate potentials in non-curarized muscles was accomplished by means of concentric electrodes which led off potential changes from the surface of the exposed *tibialis anterior*. The muscle preparation was covered with mineral oil and maintained at about 37° C by radiant heat. Details of this recording technique will be published elsewhere ([13]).

Most of the drugs were injected into the terminal end of the abdominal aorta after a preliminary ligation of the inferior mesenteric artery, the middle sacral and the contra-lateral iliac artery. Atropine and neostigmine were injected intravenously.

The drugs employed were the following:

1) Adrenaline base (synthetic).
2) Nor-adrenaline (arterenol bitartrate monohydrate, 3,4-dioxyphenyl-1-amine-2-ethanol).
3) Cobefrine hydrochloride (3,4-dioxyphenyl-1-amine-2-propanol).
4) Epinine hydrocloride (3,4-dioxyphenil-1-methylamine-2-etane).
5) Neo-synephrine hydrochloride (m-hydroxyphenyl-1-methylamine-2-ethanol).
6) Tyramine hydrcchloride (p-hydroxyphenyl-2-amine-ethane). Eastman Kodak Co.
7) Paredrine bromydrate (p-hydroxyphenil-1-amine-propane).
8) Propadrine base (phenyl-1-amine-2-propanol).
9) Amphetamine sulphate (phenyl-1-amine-2-propane).
10) Butanefrine hydrochloride (1(3,4-dioxyphenyl)-2-amine-1-butanol).
11) Isuprel sulphate (1(3,4-dioxyphenyl)-2-isopropylamine-1-ethanol).
12) Win 3046 "Winthrop" (2-isopropylamine-1-(3,4-dioxyphenyl)-butanol).

Chemical structure and doses appear in table I.

Al these substances are racemic, except nor-adrenaline which is levorotatory. Substances of catecholic structure were stabilized with sodium bisulphite in the concentrations necessary to obtain a 1 % solution.

13) d-tubocurarine (J. Berlage & Co.).
14) Decamethonium bromide (Burroughs Wellcome & Co.).
15) Neostigmine (Prostigmine "Hoffmann La-Roche" and Neostigmina "Beta").
16) Acetylcholine sulphate (Merck).*

All these drugs were dissolved in distilled water in volumes not exceeding 0.50 $cm^3$. Usually a volume of 0.10 $cm^3$ was injected. In several animals either the same drug was repeatedly injected or different drugs were administered. In any case some time was allowed to elapse between injections in order that the drugs be either eliminated or metabolized. Cyclic amines were injected at random except for butanefrine which was almost always employed before isuprel or Win 3046. The employed doses, referred to weight of the base, unless stated otherwise are given in table I. These doses produce comparable hypertension and hypotension ([16]).

## RESULTS

### 1) Effect of aromatic amines on single muscular twitch.

A) *15-17 pulses per minute.*

a) *Paredrine, tyramine, amphetamine and propadrine.* — None of these drugs had any effect (fig. 1). Under different conditions Bülbring and Burn ([6]) have demonstrated a potentiation of the muscular twitch after tyramine.

b) *Adrenaline, nor-adrenaline, cobefrine, neo-synephrine and epinine.* — Like the substances described in paragraph a), these drugs

* We wish to express our gratitude to: H. Croxatte, for 1. To Winthrop Stearn Inc. for 2, 3, 5, 10, 11 and 12. To Burroughs Wellcome Co. for 4. To Smith, Kline & French Laboratories for 7 and 9. To Parke & Davis for 8. To Instituto Bioquímico Beta for 13 and 15.

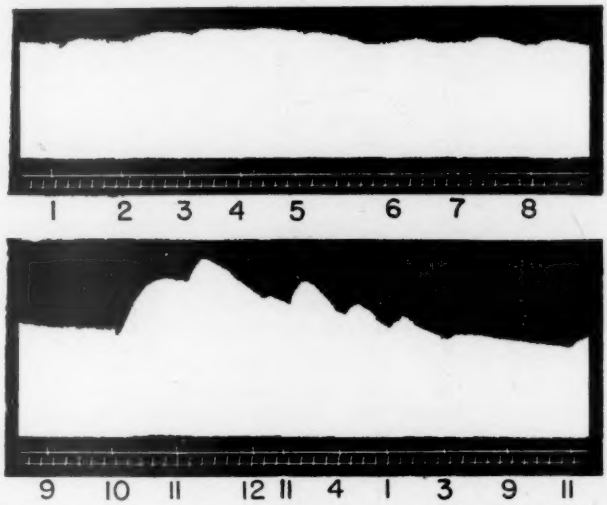



FIG. 1. — *Effects of aromatic amines on muscular twitch. Potentiating effect of neostigmine on the action of these amines. Cat weighing 3000 g anaesthetized with sodium pentobarbital. Indirect stimulation of* quadriceps femoris *at 17 pulses per minute. 1) 50 μg of neo-synephrine. 2) 250 μg of butanefrine. 3) 75 μg of isuprel. 4) 10 μg of nor-adrenaline. 5) 20 μg of nor-adrenaline. 6) 25 μg of epinine. 7) 40 μg of cobefrine. 8) 15 μg adrenaline. 9) 200 μg of paredrine. 10) 100 μg of neostigmine given intravenously. 11) 12 μg of adrenaline. 12) 20 μg of Win 3046. Time: one minute.*

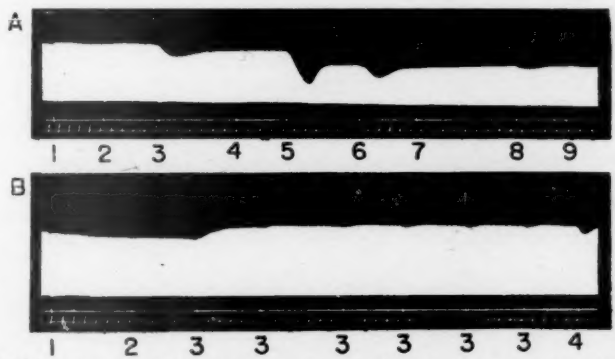
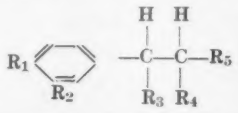



FIG. 2. — *Effect of aromatic amines on muscular twitch. Cat weighing 2200 g anaesthetized with sodium pentobarbital. Indirect stimulation of* quadriceps femoris *at 222 pulses per minute. A. 1) 75 μg of isuprel. 2) 20 μg of Win 3046. 3) 40 μg of cobefrine. 4) 250 μg of tyramine. 5) 5 μg of adrenaline. 6) 10 μg of nor-adrenaline. 7) 200 μg of paredrine. 8) 50 μg of neo-synephrine. 9) 25 μg of epinine. B. 1) 750 μg of propadrine. 2) 1000 μg of propadrine. 3) 500 μg of butanefrine. 4) 1000 μg of butanefrine. Records A and B taken in different cats of the same weight. Time: one minute.*

TABLE I

*Chemical structure and doses of aromatic amines.*



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | Doses μg |
|---|---|---|---|---|---|---|
| Win 3046 | OH | OH | OH | NH-CH-$(CH_3)_2$ | $CH_2$-$CH_3$ | 20-200 |
| Isuprel | OH | OH | OH | NH-CH-$(CH_3)_2$ | H | 75-200 |
| Butanefrine | OH | OH | OH | $NH_2$ | $CH_2$-$CH_3$ | 250-1000 |
| Nor-adrenaline | OH | OH | OH | $NH_2$ | H | 10-30 |
| Epinine | OH | OH | H | NH-$CH_3$ | H | 25-100 |
| Cobefrine | OH | OH | OH | $NH_2$ | $CH_3$ | 40-100 |
| Adrenaline | OH | OH | OH | NH-$CH_3$ | H | 5-30 |
| Neo-synephrine | | OH | OH | NH-$CH_3$ | H | 50-200 |
| Tyramine | OH | | H | $NH_2$ | H | 250-1000 |
| Paredrine | OH | | H | $NH_2$ | $CH_3$ | 100-500 |
| Propadrine | | | OH | $NH_2$ | $CH_3$ | 750-1000 |
| Amphetamine | | | H | $NH_2$ | $CH_3$ | 250-2000 |

always induced hypertension. In addition they have an action on the muscular twitch. Slight potentiation, never exceeding 10 % sometimes followed the injection of adrenaline, nor-adrenaline and cobefrine. This phenomenon lasted for a few minutes. Fig. 1 illustrates the potentiating effect of these substances. However in a few experiments there was depression or no effect. Depression of the twitch, was always less intense than the potentiation. Neo-synephrine produced irregular results i.e., either potentiation or depression. In some cases it did not modify twitch tension. Epinine had no effect. These results agree with what has been described in the literature for adrenaline ([3, 6, 8, 10] and [18]), nor-adrenaline ([10]), ephedrine, tyramine, dioxyphenilamine and sympatol ([6]).

When adrenaline was injected approximately every five minutes, tachyphylaxis was not observed. This result does not agree with that of Brown, Goffart and Dias ([4]). Crossed tachyphylaxis from adrenaline to any of the drugs described in this paragraph was not observed.

c) *Butanefrine, Win 3046 and isuprel.* — These hypotensive substances increased the strength of the muscular twitch as is illustrated in fig. 1. The character of this potentiation is similar to that observed when catecholic hypertensive amines are employed. In a few instances they did not modify the muscular response. Results obtained with butanefrine were less constant because it occasionally induced depression of twitch tension. Intensity and duration of the effects of these substances when the neuromuscular preparation was indirectly stimulated at 15-17 pulses per minute were more or less similar. In contrast, the intensity of the effects of these amines varied a great deal when frequency of stimulation was higher, as may be seen in the following paragraph.

B) *200-230 pulses per minute.*

a) *Paredrine, tyramine, amphetamine and propadrine.* — These drugs did not modify the muscular twitch (fig. 2 B). Other authors have described a similar ineffectiveness of amphetamine, though the drug may increase the response of a fatigued muscle (19).

b) *Adrenaline, nor-adrenaline, cobefrine, neo-synephrine and epinine.* — The first three drugs decreased muscular shortening. Intensity and duration of this action decreased in the following order: adrenaline, nor-adrenaline and cobefrine, as is illustrated in fig. 2 A. Wen 15 μg of adrenaline were employed, 80 % depression of the contraction was observed. This action may last over 25 minutes. Sometimes depression is preceded by small potentiation. Results obtained with adrenaline are in agreement with data available in the literature (18). Results obtained with neo-synephrine and epinine are dubious.

c) *Butanefrine, Win 3046 and isuprel.* — These chemicals either did not modify muscular tension (fig. 2 A) or increased the contraction (fig. 2 B). Butanefrine caused an ever decreasing potentiation until muscular contraction was slightly depressed (fig. 2 B).

C) *Effect of adrenaline on the end-plate potential of uncurarized muscle.*

It is known that adrenaline potentiates the responses to acetylcholine (1, 7, 9, 12 and 18). It therefore seemed important to know whether adrenaline affects the end-plate potential of uncurarized muscle. The *tibialis anterior* was used, indirectly stimulated at a frequency of 12 pulses per minute. Results illustrated in fig. 3 indicate that adrenaline increases the end-plate potential (from 2.8 mV to 3.5 mV). Also, the decaying phase of the response becomes slower than the control.

2) **A) Effects of neostigmine on the responses of aromatic amines.**

Büibring and Burn demonstrated that when the neuromuscular preparation is indirectly stimulated at a low frequency, the potentiating effect of adrenaline is increased by a previous dose of neostigmine (8). This result has been confirmed in the present study. Thus, the *quadriceps femoris* was stimulated indirectly at a frequency of 17 pulses per minute. A dose varying from 40 to 100 μg of neostigmine, given intravenously, produced a marked potentiation of the effect of adrenaline (5-20 μg) on the muscular response (fig. 1). Under the same conditions, neostigmine also potentiated the responses to nor-adrenaline, cobefrine and neo-synephrine. Examples of the action of some of these drugs are given in fig. 1. The action of neostigmine is more effective for adrenaline, and decreases in the following order: nor-adrenaline, neo-synephrine and cobefrine. Something similar has been described with adrenaline and nor-adrenaline (10). Neostigmine ocasionally potentiated the effects of epinine, butanefrine and isuprel. Win 3046 responses may also be potentiated, but this phenomenon was observed in only 3 out of 15 experiments. Paredrine and tyramine were also employed, but neostigmine had

no effect on the action of these substances. Examples of the action of some of these drugs are given in fig. 1.

**B) Effect of adrenaline on the electrogram of normal muscle before and after neostigmine.**

The *quadriceps femoris* was indirectly stimulated every 5 or every 10 seconds. Muscular contractions were recorded by means of a kymo-

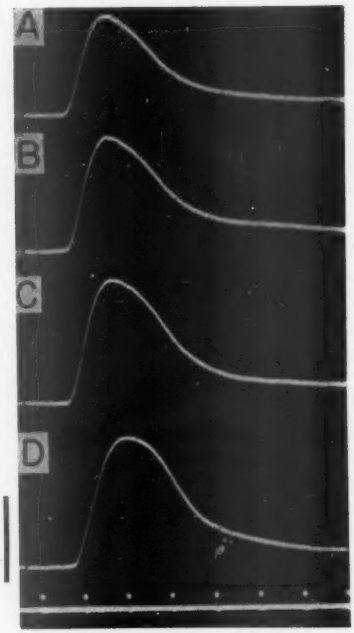



FIG. 3. — *Effect of adrenaline on the end-plate potential of uncurarized muscle. Cat weighing 2050 g anaesthetized with sodium pentobarbital.* Tibialis anterior *indirectly stimulated at a frequency of 12 pulses per minute. A. Control. B. 2 minutes after 15 μg of adrenaline injected into the aorta. C. 2 minutes after B. D. 4 minutes after. C. Cal: 2 mV. Time: 2 msec.*

graph and simultaneously the muscle action potentials were photographed from an oscilloscope. Adrenaline was injected into the aorta after a steady diphasic deflection had been observed for some time (fig. 4 A). A few seconds after the drug was injected, the responses became repetitive (fig. 4 B). Repetition lasted for a few minutes and could be abolished by increasing the rate of stimulation. This effect could be repeated several times in a single animal. The electrical response was roughly related to the dose of adrenaline, but there was no strict relation between electrical response and strength of contraction, i.e., sometimes a violent outburst of electrical activity was not accompanied by a marked increase of twitch tension. Conversely, significant potentiation of the contraction did not follow every marked repetition. Once the effect of adrenaline was over, a small dose was purposely chosen such as not to induce repetition, and if some repetitive discharges were still present, neostigmine did not modify them (fig. 4 C). When adrenaline was injected a few

minutes later, an appreciable increase of repetitive discharges was observed (fig. 4 D).

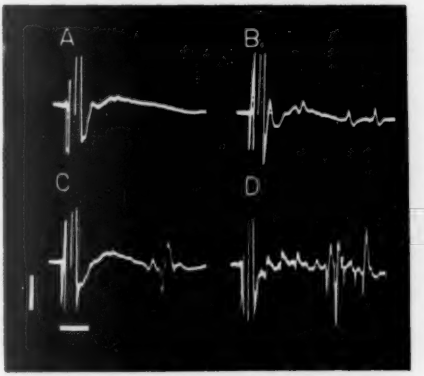



FIG. 4. — *Potentiating effect of neostigmine on the action of adrenaline on the electromyogram. Cat weighing 2000 g anaesthetized with sodium pentobarbital.* Quadriceps femoris *indirectly stimulated at a frequency of 12 pulses per minute. A. Control. B. 2 minutes after 15 μg of adrenaline. Between A and B, 10 μg of adrenaline. C. 2 minutes after 100 μg of an intravenous injection of neostigmine. D 2 minutes after 15 μg of adrenaline. Adrenaline was given 20 minutes after neostigmine. Cal: 1 mV. Time: 10 msec.*

**C) Potentiating effect of decamethonium on the activity of aromatic amines in the neuromuscular preparation.**

In cats, anaesthetized either with sodium pentobarbital or chloralose the *quadriceps femoris or the tibialis anterior* was stimulated indirectly at a frequency up to 17 pulses per minute. The dose of C-10 was from 1 μg to 15 μg injected into the aorta. Adrenaline, nor-adrenaline, cobefrine and neo-synephrine were used in these experiments. The experimental procedure was the following: the amines referred to were injected previously as control. Then 30 seconds before another injection of one of these compounds, C-10 was given. Figure 5 indicates that decamethonium increased the action of these amines. It must be emphasized that neostigmine showed to be more effective than C-10 in producing the already described effects.

**3) A) Effect of aromatic amines on neuromuscular transmission blocked by d-tubocurarine.**

Stimulus frequency ranged from 15-17 pulses per minute. Approximately 300 μg of d-tubocurarine was injected into the aorta. There followed a 90 % to 100 % reduction of the twitch tension lasting several minutes. Aromatic amines were injected at the beginning of the neuromuscular recovery. Another dose of d-tubocurarine was administered, if the twitch tension had recovered about 50 %, in order to abolish muscular shortening. In this way, several substances were tried in a single animal.

a) *Paredrine, tyramine, amphetamine and propadrine.* — Paredrine increased curarization slightly (no effect was observed in one animal out of five). The same action was observed with tyramine. Tyramine, amphetamine and propadrine did not modify twitch tension during partial curarization.

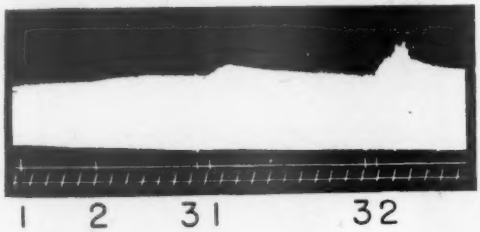



FIG. 5. — *Potentiating effect of small doses of C-10 to the action of adrenaline and nor-adrenaline on single muscular twitch. Cat weighingh 2500 g anaesthetized with sodium pentobarbital.* Quadriceps femoris *indirectly stimulated at a frequency of 17 pulses per minute. (1) 15 μg of adrenaline; (2) nor-adrenaline, 10 μg; (3) 5 μg of C-10. Time in minutes.*

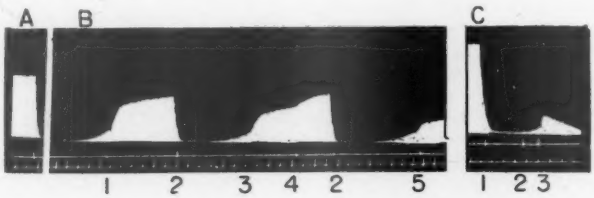



FIG. 6. — *Effects of Butanefrine, Win 3046, Isuprel, Neo-Synephrine, amphetamine and cobefrine on the neuromuscular blockade induced by d-tubocurarine. A and B: cat weighing 3300 g anaesthetized with sodium pentobarbital.* Quadriceps femoris *stimulated indirectly at a frequency of 17 pulses per minute. A. At the signal intra-aortic injection of 300 μg of d-tubocurarine. B. 10 minutes after A. 1) 250 μg of butanefrine. 2) 60 μg of d-tubocurarine. 3) 20 μg of Win 3046. 4) 75 μg of isuprel. 5) 50 μg of neo-synephrine. C. Cat weighing 2500 g anaesthetized with sodium pentobarbital.* Quadriceps femoris *stimulated at a frequency of 17 pulses per minute. 1) 116 μg of d-tubocurarine. 2) 1000 μg of amphetamine. 3) 500 μg of cobefrine. Time: one minute.*

b) *Adrenaline, nor-adrenaline and cobefrine.* — Both *quadriceps femoris* and *tibialis anterior* were used in cats anaesthetized either with sodium pentobarbital or chloralose. In all experiments these drugs had a decurarizing action. The action of cobefrine is illustrated in fig. 6 C. These findings agree with other investigations of adrenaline and nor-adrenaline ([3, 4] and [20]). The decurarizing action of these substances is not marked and it lasts about 3 minutes (fig. 6 C).

c) *Neo-synephrine, epinine, butanefrine, Win 3046 and isuprel.* — These substances showed a decurarizing effect in all the experiments (fig. 6 B). This action is not pronounced and appears slowly (fig. 6 B).

**B) Effect of aromatic amines on neuromuscular transmission blocked by decamethonium (C-10).**

Methods employed were similar to those of section A.

a) *Paredrine, tyramine, amphetamine and propadrine.* — These drugs did not show any action on the block caused by C-10.

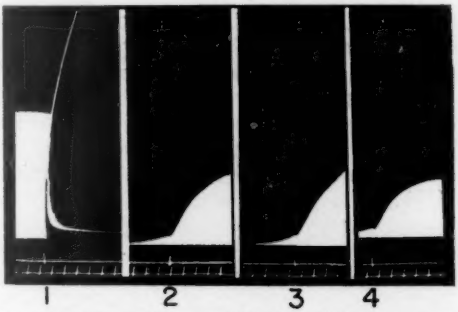



FIG. 7. — *Effects of cobefrine, epinine and butanefrine on neuromuscular blockade induced by C-10. Cat weighing 2560 g anaesthetized with sodium pentobarbital.* Quadriceps femoris *indirectly stimulated at a frequency of 17 pulses per minute. The animal had previously received 12.5 and 2.5 μg of C-10 alternating with 15 μg of adrenaline during a period of 26 minutes, before the first record. 1) 100 μg of C-10. 2) 40 μg of cobefrine. 3) 50 μg of epinine. Between 2 and 3, 65 μg of C-10. 4) 500 μg of butanefrine. Between 2 and 4, 95 μg of C-10. Time: one minute.*

b) *Adrenaline, nor-adrenaline and cobefrine.* — When the neuromuscular preparation was blocked by the action of C-10, these drugs counteracted this effect. Figure 7 illustrates the action of cobefrine. These results agree with those of West and Zaimis for the action of adrenaline and nor-adrenaline ([20]). It must be emphasized that the antagonistic action of these drugs is not constant; it was found only in about 70-80 % of the experiments. The anti-decamethonium action appears slowly (fig. 7).

c) *Epinine, neo-synephrine, butanefrine, Win 3046 and isuprel.* — These drugs in doses indicated in table I, had an anti-paralyzing effect when the neuromuscular preparation was blocked by C-10. A typical action of epinine and butanefrine is illustrated in fig. 7. The antagonistic effect of these drugs was less constant than in the case of the drugs in paragraph b). These substances showed decreasing activity, as enumerated in section c); e.g., epinine showed no effect in two of seven experiments; on the contrary, isuprel had an anti-paralyzing action in one of five experiments. Also, twitch tension reappeared slowly.

It is interesting to notice that the antagonistic activity of these amines depends both on their chemical structure and on whether the preparation was blocked by C-10 or by d-tubocurarine (table I). In both cases, benzylic amines are ineffective; hypertensive catecholic amines are less effective when antagonizing C-10 than d-tubocurarine; the remaining "hypertensive amines" are less effective than catecholic amines. "Hypotensive amines" are almost ineffective against C-10.

## Discussion

The mechanism by which adrenaline increases muscular twitch is not well understood. Some authors have suggested that this phenomenon is due to release of K [15]. Results obtained by other investigators indicate that the mechanism of action is more complex. It has been reported in several papers that adrenaline potentiates the response induced by acetylcholine [1, 7, 9, 12 and 18]. It is also known that adrenaline and similar substances counteract the blockade by d-tubocurarine and C-10 [3, 4, 20 and this paper]. These facts suggest a synaptic action, which has been confirmed by the end-plate potential changes obtained after the injection of the drug. Adrenaline was less effective against C-10 block than it was against the neuromuscular blockade after d-tubocurarine. This phenomenon may be explained by the depressant action of C-10 on the muscle fiber [17].

It has been suggested that the potentiating effect of adrenaline on muscular twitch tension is due to a direct action of the fiber for the following reasons: adrenaline may induce shortening of denervated muscles [5 and 18], modification of the action potentials [3], and the demarcation potential [4] of normal muscle. The action of adrenaline on the metabolism of muscle is beyond the scope of this paper to be referred here. Furthermore, repetitive responses have been described in this paper. Repetitive responses after neostigmine and eserine have been held responsible for the twitch potentiation when the muscle is indirectly stimulated at low frequencies [2]. By similar reasoning repetitive discharges after adrenaline could explain increased muscular shortening. The increase in mechanical response when adrenaline is given after neostigmine, may be explained by the increase of repetition which occurs under these circumstances. This is probably not the sole explanation of the facilitation of twitch tension, for a strict relation between tension developed and amount of repetition could not be established (see Results). It consequently seems clear that the action of adrenaline on the neuromuscular preparation involves both synaptic and muscular mechanisms.

Probably the mechanism of action of other aromatic amines is similar to that of adrenaline. This assumption is based on the fact that these amines have similar pharmacological effects and similar chemical structure.

It is important to emphasize here that the effect of adrenaline is more intense if this drug is injected during muscular fatigue [18]. Also, the potentiating effect of amphetamine appears only under this condition [19]. For the time being it is difficult to explain such a phenomenon. A tentative explanation could be that these compounds are capable of restoring some phase of muscle metabolism which has been depressed by fatigue.

The antagonistic effect of a few aromatic amines on paralysis induced by d-tubocurarine and C-10 are known [3, 4 and 20]. In this paper other cyclic amines have been studied. The synaptic action of adrenaline could explain the decurarizing effect of the drug. Nevertheless, the antagonistic effect against C-10 paralysis, which acts by a different mecha-

nism, needs further exploration ([21]). Probably in the latter case, actions of adrenaline beyond the end-plate are playing an important rôle.

Although the neuromuscular effects of some aromatic amines are independent of their hypertensive and hypotensive actions, the vascular changes may sometimes overshadow them ([15] and [18]). Results obtained in this paper confirm this possibility (see Results, section I A and I B). Another fact reinforces the masking effect of vascular changes on the neuromuscular action of these amines; a single injection of butanefrine induces hypotension, but repetition of the dose converts hypotension into hypertension ([11] and [16]). Under certain experimental conditions (stimulating with a frequency of about 200 pulses per minute), a single injection of butanefrine induced an increase of muscular tension, this potentiation declined and was transformed into depression when further doses of butanefrine were injected.

Apparently when the neuromuscular preparation is being stimulated with a frequency of 200 pulses per minute, vascular changes are more important in what strength of contraction is concerned. This is exemplified by the fact that twitch potentiation is obtained only after "hypotensive amines".

Aromatic amines require a special structure in order to be able to potentiate muscular contractions induced by indirect stimulation. This effect has not been observed after paredrine, tyramine, amphetamine and propadrine. In order to obtain maximal potentiation, two structural conditions seem to be required: a catecholic ring (3,4-hydroxybenzyl) and one hydroxyl group on the alpha carbon of the lateral chain. The transformation of the phenylic into a benzylic ring produces inactive compounds such as propadrine and tyramine. These structural conditions are also necessary to counteract the neuromuscular block induced by d-tubocurarine and C-10 and to obtain a potentiating effect after neostigmine (Table I).

## Summary

Cats anaesthetized with sodium pentobarbital and chloralose were used throughout the experiments. *Quadriceps femoris* and *tibialis anterior* were used and stimulated indirectly.

1. — Adrenaline, nor-adrenaline, cobefrine, butanefrine, isuprel and Win 3046 potentiated the twitch tension slightly when a frequency of stimulation up to 17 pulses per minute was used. Neo-synephrine gave inconclusive results. Epinine had no action. Adrenaline, nor-adrenaline and cobefrine depressed muscular contractions when the neuromuscular preparation was stimulated at a frequency of 200 pulses per minute, Win 3046, isuprel and butanefrine increased muscular shortening when studied using the same frequency of stimulation.

Paredrine, amphetamine, tyramine and propadrine did not modify muscular contractions.

2. — Neostigmine increased potentiation induced by adrenaline, nor-adrenaline, cobefrine, neo-synephrine and epinine (low frequency stimulation). Neostigmine sometimes potentiated the effects of butanefrine,

isuprel and Win 3046 but it had no action on the effects of paredrine and tyramine.

3. — Decamethonium increased the effect of adrenaline, nor-adrenaline, cobefrine and neo-synephrine when the neuromuscular preparation was stimulated at 15-17 pulses per minute.

4. — During the twitch potentiation induced by adrenaline, the end-plate potential became larger and more prolonged; also, repetitive responses were observed. Repetitive discharges induced by adrenaline were potentiated by neostigmine.

5. — Adrenaline, nor-adrenaline, cobefrine, epinine, butanefrine, isuprel and Win 3046 antagonized the neuromuscular block induced by d-tubocurarine. Paredrine, tyramine, amphetamine and propadrine were ineffective. Adrenaline, nor-adrenaline, cobefrine, epinine, neo-synephrine and butanefrine counteracted the blocking action of C-10 on neuromuscular transmission. Isuprel and Win 3046 were occasionally effective. Paredrine, tyramine, amphetamine and propadrine were ineffective.

6. — The mechanism by which adrenaline increases muscular twitch tension is discussed.

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## REFERENCES

(1) BENÍTEZ, D., HERREROS, M., MIDDLETON, S., TALESNIK, J.: *Bol. Soc. Biol., Santiago, Chile.* 1948-1949, *6*, 86.
(2) BROWN, G. L.: *J. Physiol.*, 1937, *89*, 220.
(3) BROWN, G. L., BÜLBRING, E., BURNS, B. D.: *J. Physiol.*, 1948, *107*, 115.
(4) BROWN, G. L., GOFFART, M., DIAS, M. V.: *J. Physiol.*, 1950, *111*, 184.
(5) BÜLBRING, E., BURN, J. H.: *J. Physiol.*, 1936, *86*, 61.
(6) BÜLBRING, E., BURN, J. H.: *J. Pharmacol. exper. Therap.*, 1940, *68*, 150.
(7) BÜLBRING, E., BURN, J. H.: *J. Physiol.*, 1941, *100*, 337.
(8) BÜLBRING, E., BURN, J. H.: *J. Physiol.*, 1942, *101*, 224.
(9) BÜLBRING, E., BURN, J. H.: *J. Physiol.*, 1942, *101*, 289.
(10) BURN, J. H., HUTCHEON, D. E.: *Brit. J. Pharmacol.*, 1949, *4*, 373.
(11) CAMERON, W. M., WHITSELL, L. J., CRISMON, J. H., TAINTER, M. L.: *J. Pharmacol. exper. Therap.*, 1938, *63*, 340.
(12) DANNA, M., MIDDLETON, S., TALESNIK, J.: *Bol. Soc. Biol. Santiago, Chile*, 1948-1949, *6*, 18.
(13) EYZAGUIRRE, C.: (in preparation).
(14) EYZAGUIRRE, C., FOLK, B. P., ZIERLER, K. L., LILIENTHAL, J. L. JR.: *Am. J. Physiol.*, 1948, *155*, 69.
(15) GOFFART, M., BROWN, G. L.: *C. R. Soc. Biol. Paris*, 1947, *141*, 958.
(16) HUIDOBRO, F., CROXATTO, R., ALLENDE, J., DEL RÍO, J.: *Acta Physiol., Lat.-Amer.*, 1951, *1*, 91.
(17) JARCHO, L. W., EYZAGUIRRE, C., TALBOT, S. A., LILIENTHAL, J. L., JR.: *Am. J. Physiol.*, 1950, *162*, 475.
(18) LUCO, J. V.: *Am. J. Physiol.* 1939, *125*, 196.
(19) LUCO, J. V.: EYZAGUIRRE, C., PÉREZ, F.: *J. Pharmacol. exper. Therap.*, 1948, *93*, 261.
(20) WEST, G. B., ZAIMIS, E. J.: *J. Physiol.*, 1949, *110*, 18 P.
(21) ZAIMIS, E. J.: *J. Physiol.*, 1951, *112*, 176.

# INTERVENCIÓN DE CORTEZA CEREBRAL EN LA INTEGRACIÓN DE UNA REACCIÓN VEGETATIVA

## Condicionamiento de un estímulo táctil, a la acción del cianuro.

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LOS TRABAJOS de nuestro laboratorio están encaminados al estudio de los mecanismos nerviosos que controlan las funciones responsables de la estabilidad del medio interno en los mamíferos superiores. La mayor parte de los experimentos hechos con esta finalidad, tanto por nosotros como por otros autores, fueron "agudos" y realizados en animales anestesiados, en los cuales quedan eliminadas las funciones correspondientes a los centros nerviosos superiores, fundamentalmente el telencéfalo.

Como es lógico sospechar que en condiciones fisiológicas el telencéfalo intervenga en la integración de las respuestas que surgen como consecuencia de variaciones en el medio interno, decidimos iniciar estudios en animales que conservaran sus funciones telencefálicas.

En los perros sin anestesiar pueden surgir cambios en la frecuencia cardíaca y en la respiración, como consecuencia de un sinnúmero de estímulos incontrolados que llegan hasta el animal, por lo que es necesario mantenerlo en las condiciones de aislamiento, imprescindibles para asegurarnos de que las reacciones que se obtengan sobre respiración o frecuencia cardíaca son respuestas a los estímulos que nos proponemos aplicar.

Estas condiciones pueden conseguirse siguiendo el método habitual para el estudio de reflejos condicionados, por lo cual decidimos montar una cámara que nos permitiera desarrollar este tipo de estudios [1].

El cambio del medio interno que seleccionamos como estímulo, fué la variación del oxígeno arterial, debido a su importancia fisiológica y a que disponemos de datos directos del lugar y forma en que actúa este estímulo (función quimiorreceptora, en las zonas reflexogénicas [2, 3, 4]).

Como los efectos del cianuro son equivalentes a la anoxia anoxémica, decidimos utilizar inyecciones de cianuro como estímulo directo. Para hacer llegar hasta los interorreceptores la solución de cianuro sin producir ningún estímulo adicional como consecuencia de la entrada del investigador a la cámara del animal o de la manipulación necesaria para inyec-

tar, se ideó un sistema hidráulico que permite inyectar en cualquier momento desde la cámara del observador.

Para el registro de la función cardíaca se utilizó un electrocardiógrafo, construído especialmente en nuestro laboratorio, que nos proporciona el trazo directamente en el quimógrafo, simultáneamente con el registro de la respiración.

En la cámara se dispone de los aditamentos necesarios para la aplicación de estímulos táctiles, eléctricos, visuales y auditivos. En la figura 1 aparece un esquema de esta cámara, con los dispositivos especiales que fueron montados para este trabajo.

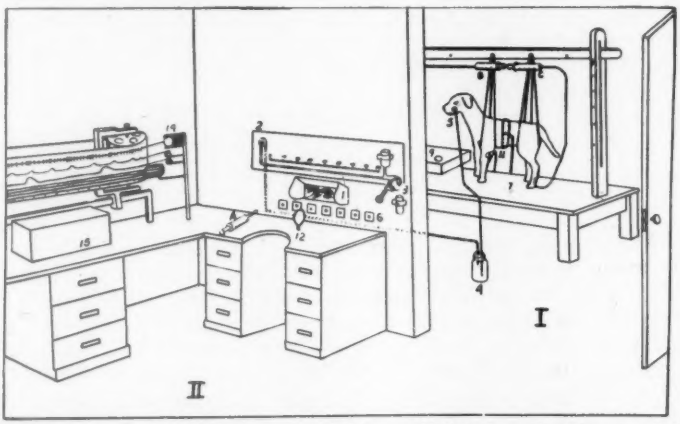


FIG. 1. — *Esquema de la cámara de reflejos condicionados. I, Cámara del animal. II, Cámara del observador. 1) Mirilla; 2) Manómetro de saliva; 3) Contador de gotas de saliva; 4) Colector de saliva; 5) Embudo para colectar saliva; 6) Interruptores eléctricos que controlan las excitaciones: luminosa, sonora, alimenticia, dolorosa; 7) Mesa; 9) "Comedero"; 11) Estímulo táctil; 12) Pera de goma para accionar el estímulo táctil; 14) Pajilla del electrocardiógrafo; 15) Electrocardiógrafo.*

En las condiciones de aislamiento de esta cámara, se observó en el electrocardiograma de todos los perros, una arritmia respiratoria muy acentuada, la cual aparece con mayor claridad cuando el perro está tranquilo o dormido, lo que nos decidió a tomarla como indicador del grado de reposo del animal y de haber cesado todas las reacciones de orientación que presenta el perro al estar en un medio nuevo para él.

En los perros anestesiados con nembutal, cloralosa, uretano, etc. (5), se comprobó que el cianuro produce bradicardia refleja; sin embargo, las inyecciones de cianuro hechas a perros sin anestesiar producen una taquicardia definida como la que se presenta en la figura 2. La intensidad de la misma es función de las dosis inyectadas. Con objeto de determinar la relación dosis-efecto, se hizo una serie de observaciones con cuyos datos se construyó la curva que aparece en la figura 3. De su análisis se deduce que la dosis umbral es aproximadamente de 0.02 mg por kg de peso, y que el efecto que ocasiona va aumentando al aumentar la cantidad de

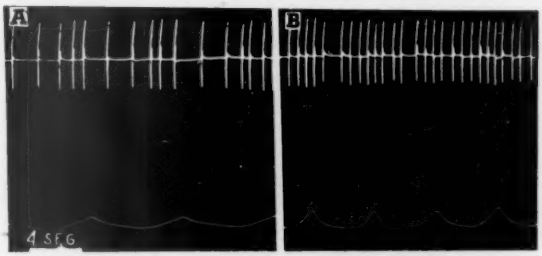


FIG. 2. — *Perro en la cámara de reflejos condicionados. Efectos sobre frecuencia cardíaca y respiración, de la inyección intraperitoneal de 200 μg de cianuro por kg de peso corporal.* A, *antes de la inyección;* B, *después de la inyección.*

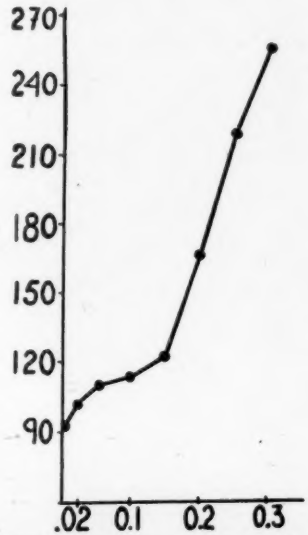


FIG. 3. — *Curva que relaciona los cambios de frecuencia cardíaca con distintas dosis de cianuro. En las ordenadas, frecuencias cardíacas en pulsaciones por minuto; en las abscisas, cantidad de cianuro inyectado en mg por kg de peso.*

cianuro inyectado. Examinando el desarrollo en el tiempo de las respuestas producidas por 0.2 mg, inyectados por vía intraperitoneal, vimos que la latencia oscilaba entre uno y dos minutos, siendo en la mayor parte de los casos de 1 m 20 seg. La frecuencia cardíaca alcanza su máximo a los 3.5 m, y varía entre el 170 y el 240 % de su valor inicial.

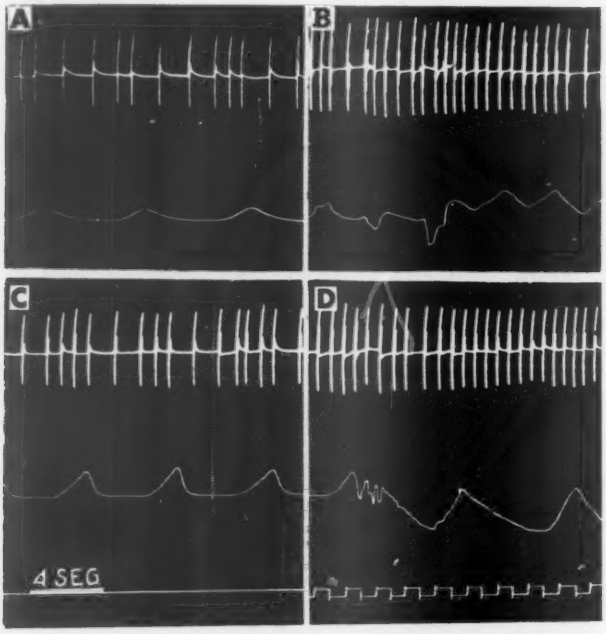


FIG. 4. — *Perro en la cámara de reflejos condicionados. Efectos de la inyección intraperitoneal de cianuro y efectos de un estímulo táctil condicionado.* A, *antes de la inyección de cianuro.* B, *Después de la inyección de cianuro.* C, *Antes de la aplicación del estímulo táctil condicionante.* D, *Después del estímulo táctil condicionante. En este último registro, además de electrocardiograma y respiración, aparece la señal electromagnética que marca los momentos de aplicación intermitente del estímulo.*

El hecho de ser la respuesta a la inyección, en los animales sin anestesiar, la inversa de la obtenida en los animales anestesiados, nos planteó el problema de si esta diferencia en la reacción es debida a la intervención de los centros telencefálicos, que quedan eliminados en el estado de anestesia, o si dependen de los efectos del anestésico sobre los centros del bulbo raquídeo.

Uno de los procedimientos que pueden utilizarse para estudiar la intervención de corteza cerebral en la integración de estas respuestas, es intentar la formación de reflejos condicionados. Con ese fin se planeó una serie de experimentos en los cuales intentamos condicionar el efecto

que produce el cianuro, en los animales sin anestesiar, a un estímulo táctil ligero.

Se escogió para ello un perro que, por su conducta, debía clasificarse según los criterios de la escuela de Pavlov, como sanguíneo. Se le inyectó cianuro aplicando a continuación un estímulo táctil y tomando los registros de respiración y electrocardiograma simultáneamente. El estímulo táctil produjo respuestas sobre corazón y respiración, con signos claros de condicionamiento (figura 4).

Se escogió para su análisis la reacción sobre corazón porque tomando como índice la frecuencia cardíaca, se puede obtener datos cuantitativos

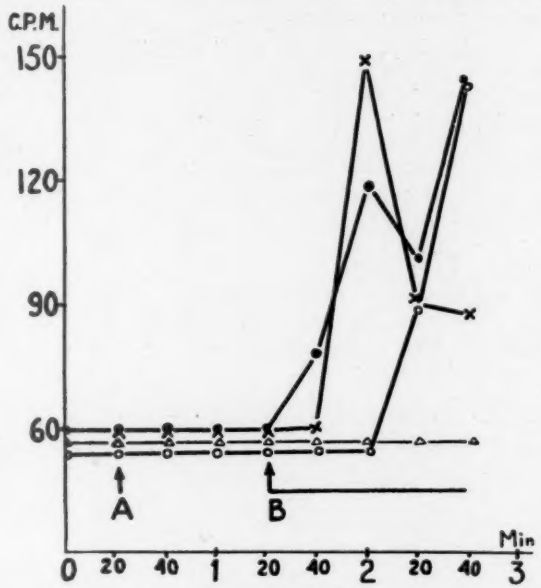


FIG. 5. — *Perro en la cámara de reflejos condicionados. Desarrollo en el tiempo del efecto que sobre frecuencia cardíaca produce:* ○ *200 μg por kg de peso de cianuro,* △ *el estímulo táctil antes de condicionarse;* × *después de condicionarse y* • *la aplicación simultánea del estímulo táctil con la inyección de cianuro después del condicionamiento. Ordenadas: frecuencia cardíaca en ciclos por minuto. Abscisas: tiempo en minutos.*

fáciles de comparar, mientras que en el registro de respiración hemos observado que la respuesta se manifiesta en algunas ocasiones por un aumento de frecuencia, en otras por un aumento de amplitud y en muchas se mezclan ambos componentes.

En algunos experimentos hubo signos obvios de condicionamiento en reacciones del animal, de las cuales no podíamos obtener registro gráfico y mucho menos datos cuantitativos; por ejemplo, sus muestras de intranquilidad, una forma peculiar de respiración con la boca abierta, hipersalivación, etc., que se presentan con el estímulo táctil en forma semejante a como aparecen después de la inyección de cianuro.

Para el estudio analítico de las respuestas condicionadas sobre frecuencia cardíaca, se construyeron las curvas de la figura 5, colocando en las ordenadas la frecuencia cardíaca en pulsaciones por minuto, y en las abscisas, el tiempo. En *A*, la inyección de cianuro, en *B*, la aplicación del estímulo táctil. La curva que une los puntos marcados con círculos describe el desarrollo en el tiempo que sobre frecuencia cardíaca producen los 200 microgramos de cianuro inyectados por vía intraperitoneal. La curva que une los puntos marcados con triángulos demuestra que el estímulo táctil, antes de su condicionamiento, no produce variaciones de frecuencia cardíaca. La que une los puntos marcados con triángulos negros representa el efecto que produce este mismo estímulo después de su condicionamiento.

Estas curvas demuestran claramente que después del condicionamiento el estímulo táctil produce un efecto similar a la inyección de cianuro, tanto en lo que respecta a intensidad de la respuesta como a su desarrollo temporal. Cuando se aplican simultáneamente el estímulo directo y el condicionante, se obtiene una respuesta que corresponde a la curva que une los círculos negros en la figura 5. Del análisis de esta curva se desprende que al aplicar simultáneamente el estímulo condicionante y el directo, la respuesta es iniciada por el estímulo condicionante, mientras que el mantenimiento del efecto es debido al estímulo directo.

El hecho de simular el estímulo táctil la presencia de cianuro en sangre, demuestra con claridad la intervención de la corteza cerebral en la integración de estas respuestas vegetativas, en forma similar a como Buikov y sus colaboradores la han demostrado en otros mecanismos también vegetativos. Hechos que nos hacen ver, que no podemos limitar el estudio de estas funciones o de la influencia que sobre ellas ejercen algunas substancias, a los datos que nos proporcionan los experimentos "agudos", hechos en animales anestesiados, pues en tales casos las respuestas son más esquemáticas por no intervenir en ellas el órgano que en los mamíferos superiores tiene la función de integrar, a un nivel superior, tanto los componentes somáticos como los componentes vegetativos de las reacciones que aparecen en respuesta a un cambio en el equilibrio del medio interno.

## BIBLIOGRAFÍA

(1) ALVAREZ-BUYLLA, R.: Descripción de una cámara para el estudio de reflejos condicionados, construída en la Escuela Nacional de Ciencias Biológicas del I. P. N. *Ciencia*, 1950, *10*, 291.

(2) ALVAREZ-BUYLLA, R.: Estudio oscilográfico de la actividad eléctrica de los quimiorreceptores del seno carotídeo en el perro. *Acta Physiol. Lat.-Amer.*, 1951, *1*, 11.

(3) ALVAREZ-BUYLLA, R.: Estudio cuantitativo de la actividad quimiorreceptora del seno carotídeo del perro. (En prensa.)

(4) ALVAREZ-BUYLLA, R.: Análisis experimental de la función del nervio aórtico *(N. Depressoris)*. *Mem. Rev. Acad. Nac. Cienc.*, 1949, *56*, 383.

(5) ALVAREZ-BUYLLA, R.: Influencia de la actividad de los quimiorreceptores del seno carotídeo, sobre la frecuencia cardíaca. *Anal. Esc. Nac. C. Biol.*, 1949, *6*, 175.